tion is effective only for PD-1^{lo} and not PD-1^{hi}, which are subsets of exhausted T cells (50). Many of these inhibitory receptors are either coexpressed by the same exhausted T cells or differentially expressed on different subsets of exhausted cells. As the severity of the infection increases, the number of different inhibitors expressed per cell increases (47). A second inhibitory receptor, CTLA-4, can be overexpressed by exhausted CD4$^+$ T cells in chronic viral infection, but it appears to have a minimal role on exhausted CD8$^+$ T cells (19, 51). Although CTLA-4 was only slightly upregulated on CD8$^+$ T cells in contrast to the marked upregulation of PD-1 in our CD8-dependent model of MHC-matched BMT, the precise inhibitory receptors of therapeutic interest may differ between CD4$^+$-dependent and CD8$^+$-dependent GVHD/GVL. Another key negative regulatory pathway is mediated by Foxp3$^+$ Tregs. However, enhancement of GVL is not due to effects of the PD-1/PD-L1 blockade on Tregs, because blockade of PD-1/PD-L1 interactions enhances the expansion and function of Tregs (52). The hierarchy of these pathways in regulating GVL will need to be studied in the future based on better understanding of the delineation of T cell subsets and models (53). However, our results suggest the detrimental effect of GVHD-induced immunosuppression on GVL responses, regardless of which inhibitory pathway might be dominant clinically.

In addition, the administration of anti-PD-L1 mAb also exacerbated acute GVHD, as has been shown in a previous study (54). Therefore, the beneficial effects of the PD-1/PD-L1 blockade may be offset by the exacerbation of GVHD. Effects of the inhibitory receptor blockade might depend on the magnitude or stage of donor T cell activation and the severity of GVHD; therefore, the timing and duration of the targeting may be important.

In clinical HSCT, alloantigens continue to be presented on MHC class I in non-hematopoietic cells throughout the lifetime of the transplant recipients. However, a substantial number of patients eventually develop tolerance after resolution of GVHD and often experience leukemia relapse. Although activation-induced apoptosis of alloreactive T cells has been proposed as an explanation of this paradox (55), studies monitoring GVHD-specific T cell clones indicate that host-reactive T cells are continuously present after allogeneic HSCT (56–58). Our results provide a logical explanation for this paradox. However, the process of exhaustion is unlikely to occur in patients not developing GVHD, because induction of T cell exhaustion requires antigen-specific activation of T cells and subsequent differentiation into effector T cells. In these patients, tolerance could be induced by other mechanisms, such as functional central and peripheral tolerance mechanisms. It is well known that GVL is not apparent in patients with high leukemia burden. Although leukemia cells used in the current study do not express PD-L1 (22, 59), leukemia cells expressing PD-L1 may also directly limit the GVL response in patients with high leukemia burden (22, 24, 25). However, such insights from animal models must be extrapolated with caution to clinical studies involving humans.

It has been assumed that T cell exhaustion is antigen specific in chronic viral infection. Bystander lysis of T cells has also been reported in the course of viral infections (60), but is of minimal significance because of its limited magnitude and because normal thymic function can replenish the peripheral T cell pool. In contrast, in GVHD, T cell exhaustion occurs after initial T cell activation and the subsequent development of GVHD. GVHD induces bystander apoptosis of non-host-reactive T cells. In addition, GVHD-mediated injury of the thymus and the secondary lymphoid organs inhibits full replenishment of the peripheral T cell pool (55). Thus, establishment of full immune competence probably requires the additional process of T cell reconstitution following T cell exhaustion.

In conclusion, our results indicated the significance of alloantigen expression on non-hematopoietic cells in GVL. Alloantigen expression on non-hematopoietic cells induces the apoptosis of donor T cells and the dysfunction of cytotoxic effector function, which leads to a reduction in GVL activity. T cell dysfunction was partially restored by blocking PD-1/PD-L1 interactions, which suggests that the therapeutic "tuning" of T cell responses via modulation of negative regulatory pathways represents a novel strategy for enhancing GVL. Our results in combination with those of previous studies (6, 7, 9, 10, 38, 39) provide a complete picture of the effect of alloantigen expression on host APCs, GVHD target epithelium, and tumor cells in allogeneic HSCT; alloantigen expression on host non-hematopoietic cells augments GVHD but suppresses GVL effects. This concept may provide an important framework for understanding the pathophysiology of GVHD and allow for the separation of GVHD and GVL.

Methods

Mice. Female C57BL/6 (B6, H-2^b, CD45.2$^+$), BALB/c (Ba, H-2^d), and DBA/2 (Db, H-2^d) mice were purchased from Charles River Japan. B6.Ly5.1 (H-2^b, CD45.1$^+$) and C3H.Sw (C3, H-2^b) mice were purchased from The Jackson Laboratory. B6-background β_2m-deficient mice ($\beta_2m^{-/-}$: B6.129-β_2-m^{tm1Jae}N12) were purchased from Taconic. The age of mice used ranged from 8 to 12 weeks. Mice were maintained in specific pathogen-free conditions and received normal chaw and hyperchlorinated drinking water for the first 3 weeks after BMT. All experiments involving animals were performed according to a protocol approved by the Institutional Animal Care and Research Advisory Committee of Okayama University and Kyushu University.

Generation of bone marrow chimera and induction of GVHD and GVL. Total body irradiation (TBI: X-ray) was split into 2 doses separated by 4 hours to minimize gastrointestinal toxicity. B6 and C3 mice received 10 Gy TBI, whereas Ba and Db mice received 8.5 Gy TBI. To create BM chimeras, lethally irradiated mice were intravenously injected with 5×10^6 TCD BM cells from donors. TCD was performed using anti-CD90 microbeads and AutoMACS (Miltenyi Biotec). Four months later, the chimeric mice were reirradiated and injected with 5×10^6 TCD BM cells plus various doses of CD8$^+$ T cells or 2×10^6 T cells. T cells and CD8$^+$ T cells were negatively isolated from splenocytes by using a T cell isolation kit and a CD8$^+$ T cell isolation kit (Miltenyi Biotec), respectively, and the AutoMACS. In the GVL experiments, EL4 (H-2^b) derived from a B6 mouse, P815 (H-2^d) derived from a Db mouse, and A20 (H-2^d) derived from a Ba mouse were intravenously injected into BMT recipients on day 0 of BMT. Anti-PD-L1 mAbs were purified from the hybridoma supernatant of clone MIH5 (61), which was a gift from Miyuki Azuma of Tokyo Medical and Dental University, Tokyo, Japan, and i.p. injected at a dose of 500 μg/mouse on day 0, followed by 200 μg/mouse on days +3, +6, +9, +12, +15, and +18 after BMT.

Assessment of GVHD and GVL effects. Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly by using a scoring system that sums changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as described previously (13). The cause of each death after BMT was determined by post mortem examination, and was either GVHD or tumor. The most striking leukemia-specific abnormality induced by EL4, P815, and A20 was macroscopic tumor nodules, marked hepatosplenomegaly, and lower limb paralysis (62). Leukemia death induced by EL4, P815, and A20 was therefore defined by the occurrence of hepatosplenomegaly, macroscopic tumor nodules in the liver

and/or spleen, or hind leg paralysis. GVHD death was defined as the absence of leukemia and by the presence of clinical signs of GVHD, assessed by using a clinical scoring system. Animals surviving beyond the observation period of BMT were sacrificed, and the spleen and liver were harvested for histological evaluation to determine leukemia-free survival.

Flow cytometric analysis. The mAbs used were FITC-, PE-, PerCP-, Cy5.5-, or APC-conjugated anti-mouse CD5.1, CD8, CD45.1, CD45.2, CD69, and PD-1 (BD Biosciences). Cells positive for 7-amino-actinomycin D (BD Biosciences) were excluded from the analysis. For the analysis of donor T cell apoptosis, the cells were stained with Annexin V (MBL). For intracellular IFN-γ staining, the splenocytes were incubated for 4 hours with leukocyte activation cocktail and BD GolgiPlug (BD Biosciences) at 37°C. Then, the cells underwent permeabilization with a BD Cytofix/Cytoperm solution (BD Biosciences) and were stained with FITC-conjugated anti–IFN-γ mAbs (BD Biosciences). For intracellular CTLA-4 staining, cells were stained with PE-conjugated anti–CTLA-4 mAbs (eBioscience). At least 5,000 live events were acquired for the analysis using a FACSCalibur flow cytometer (BD Biosciences).

CTL assay. Splenocytes were removed from chimeric recipients 14 days after BMT, and the mononuclear cells were then separated by density gradient centrifugation. The percentage of $CD8^+$ cells in this fraction was determined by flow cytometry, and counts were normalized for $CD8^+$ cell numbers. Tumor targets, 2×10^6 P815 or EL4, were labeled with 100 μCi of ^{51}Cr sodium salt (Amersham Biosciences) for 2 hours. After washing 3 times, the labeled targets were resuspended in 10% FCS in RPMI and plated at 10^4 cells per well in U-bottom plates (Corning-Costar Corp.). Allogeneic splenocyte preparations, as described above, were added to quadruplicate wells at varying effector-to-target ratios and incubated for 4 hours. Maximal and background release were determined by adding 1% SDS and media alone to the targets, respectively. ^{51}Cr activity in the supernatants collected 4 hours later was determined using a Wallac 1470 WIZARD Gamma Counter (Wallac Oy), and lysis was expressed as a percentage of maximum: percentage of specific lysis = 100 (sample count − background count/maximum count − background count).

Quantitative real-time PCR. Total RNA was isolated from the frozen liver using ISOGEN (Nippon Gene). cDNA was synthesized from 150 μg RNA using a QuantiTect Reverse Transcription Kit (QIAGEN). *Pdl1* mRNA levels were quantified by real-time PCR using the 7500 Real-Time PCR System (Applied Biosystems). TaqMan Universal PCR MasterMix, primers, and the fluorescent TaqMan probe specific for murine PD-L1 (Mm00452054-m1) and a house keeping gene, mGAPDH (Mm99999915-g1), were purchased from Applied Biosystems. The standard was obtained using RNA extracted from syngeneic controls.

Immunohistochemistry. For immunohistochemical analysis, isolated livers were frozen in Tissue-Tek (Sakura Finetek), and 5-μm cryostat sections were prepared. Slides were fixed in 100% acetone and air dried. Endogenous peroxidase activity was blocked with peroxidase blocking reagent (Dako). The sections were incubated with purified rat anti-mouse PD-L1 mAb (clone MIH5; eBiosciences). The primary Abs were detected using the Histofine Simple Stain Mouse MAX PO (Rat) kit and DAB solution (Nichirei). The images were captured using an Olympus BH2 microscope with a Nikon DS-5M color digital camera (Nikon), controlled by Nikon ATC-2U software version 1.5. An Olympus ×10/20 ocular lens and a ×20/0.46 NA objective lens were used. Images were cropped using Adobe Photoshop (Adobe Systems) and were composed using Adobe Illustrator.

Statistics. We used the Kaplan-Meier product-limit method to obtain survival probability and the log-rank test to compare survival curves. The Mann-Whitney *U* test was used to analyze the clinical scores. A *P* value less than 0.05 was considered statistically significant.

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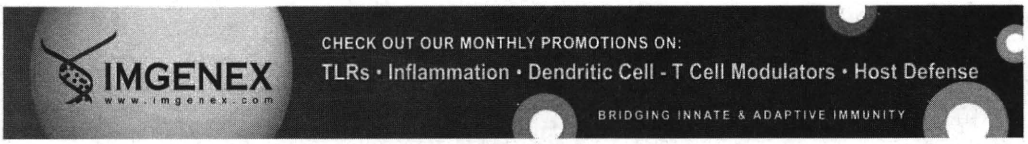

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Altered Effector CD4$^+$ T Cell Function in IL-21R$^{-/-}$ CD4$^+$ T Cell-Mediated Graft-Versus-Host Disease

Iekuni Oh,* Katsutoshi Ozaki,* Akiko Meguro,* Keiko Hatanaka,* Masanori Kadowaki,† Haruko Matsu,* Raine Tatara,* Kazuya Sato,* Yoichiro Iwakura,‡ Susumu Nakae,§ Katsuko Sudo,¶ Takanori Teshima,† Warren J. Leonard,‖ and Keiya Ozawa*

We previously showed that transplantation with *IL-21R* gene-deficient splenocytes resulted in less severe graft-versus-host disease (GVHD) than was observed with wild type splenocytes. In this study, we sought to find mechanism(s) explaining this observation. Recipients of donor CD4$^+$ T cells lacking IL-21R exhibited diminished GVHD symptoms, with reduced inflammatory cell infiltration into the liver and intestine, leading to prolonged survival. After transplantation, CD4$^+$ T cell numbers in the spleen were reduced, and MLR and cytokine production by CD4$^+$ T cells were impaired. These results suggest that IL-21 might promote GVHD through enhanced production of effector CD4$^+$ T cells. Moreover, we found that CD25 depletion altered neither the impaired MLR in vitro nor the ameliorated GVHD symptoms in vivo. Thus, the attenuated GVHD might be caused by an impairment of effector T cell differentiation itself, rather than by an increase in regulatory T cells and suppression of effector T cells. *The Journal of Immunology*, 2010, 185: 1920–1926.

Interleukin-21 was discovered as a costimulatory cytokine for T cell proliferation and NK cell expansion in vitro (1, 2). IL-21 is produced by activated CD4 T cells (1), and its receptor is expressed on T, B, and NK cells (1, 3). It was also reported that IL-21 suppresses dendritic cell function (4) and increases hematopoietic progenitor cells (5). IL-21 is known to play critical roles in Ig production (6), whereas reports have differed regarding its contributions to Th1-, Th2-, and Th17-mediated effects and differentiation (6–15). IL-21 contributes to Th17 differentiation, but it may not be required for this process (7, 9, 14, 15). A relationship between IL-21 and autoimmune disease has been established. Overexpression of IL-21 induces inflammation, and in a systemic lupus erythematosus model mouse (the BXSB.6-Yaa$^+$/J) with high serum levels of IL-21 (16), the development of disease is abrogated when these mice are crossed to IL-21R knockout (KO) mice (17). In addition, autoimmune NOD mice do not develop diabetes in the absence of IL-21 signaling (18–20).

Graft-versus-host disease (GVHD) is a major complication following hematopoietic stem cell transplantation (21), sometimes with a fatal outcome. Previously, we showed that transplantation with *IL-21R* gene-disrupted splenocytes resulted in less severe GVHD than was seen with wild type (WT) splenocytes (22). We sought to elucidate the mechanism(s) for this observation; in this article, we demonstrate dysregulated effector function of activated CD4$^+$ T cells in IL-21R$^{-/-}$ mice.

Materials and Methods

Mice

IL-21R$^{-/-}$ and IL-17$^{-/-}$ mice were generated previously (6, 23). Both were on a C57BL/6 background. Male and female mice were used as donors. C57BL/6-DBA2-F1 male mice were purchased from Clea Japan (Tokyo, Japan). All mice used in experiments were 6–12 wk old. All mice were housed in a Jichi Medical University mouse facility, which is regulated by an intramural small animal committee, and were treated in accordance with university guidelines.

In vitro T cell stimulation and MLR

Cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine (Invitrogen), 50 μM 2-ME (Sigma-Aldrich), 0.1 mg/ml streptomycin, and 100 U/ml penicillin G (Invitrogen). Nonspecific pan T cell stimulation was performed using anti-CD3/CD28 beads for 3 d, according to the manufacturer's instructions (Dynal Biotech, Oslo, Norway). Alloantigen-specific T cell stimulation was induced by cocultivation of CD4 T cells with 30 Gy-irradiated splenocytes from C57BL/6-DBA2-F1 mice for 4 d.

GVHD models

We used IL-21R$^{-/-}$ bone marrow (BM) to eliminate the effects of WT T cells in BM. We compared transplantations with IL-21R$^{-/-}$ CD4$^+$ T cells versus WT CD4$^+$ T cells. C57BL/6-DBA2-F1 mice were irradiated with 11 Gy and injected i.v. with 5×10^6 IL-21R$^{-/-}$ BM and 5×10^6 purified CD4$^+$ T cells from WT or IL-21R$^{-/-}$ mice. The cells were purified using CD4 microbeads and AutoMACS (Miltenyi Biotec, Tokyo, Japan); the purity was >80–90%.

Pathological analysis

Two weeks after transplantation, mice were sacrificed; liver, skin, and intestine were subjected to formalin fixation, paraffin embedding, excision, and H&E staining. Photographs were taken with an Olympus BX51 microscope at ×400 magnification.

Flow cytometric analysis

Fc-block (BD Biosciences, San Jose, CA) was used to prevent nonspecific Ab binding to Fc receptors. Abs to CD4 (RM4-5), CD8 (Ly-2), CD25

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The online version of this article contains supplemental material.

Abbreviations used in this paper: BM, bone marrow; GVHD, graft-versus-host disease; KO, knockout; Treg, regulatory T; WT, wild type.

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(7D4), H-2^b (AF6-88.5), H-2^d (SF1-1.1), IFN-γ (XMG1.2), and TNF-α (MP6-XT22) were purchased from BD Biosciences, and anti-Foxp3 (FJK-16a) was from eBioscience (San Diego, CA). Intracellular staining was performed with a Cytofx/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. Cells were stimulated with anti-mouse CD3/CD28 beads for 5 h in the presence of GolgiStop (BD Biosciences). The stimulation was omitted for Foxp3 intracellular staining. An LSR flow cytometer (BD Biosciences) was used for data collection, and data were analyzed using CellQuest software (BD Biosciences).

ELISA

ELISA kits for IL-2, IL-4, and IFN-γ were from BD Biosciences, and ELISA kits for IL-21, IL-17, TNF-α, and TGF-β1 were from R&D Systems (Minneapolis, MN). Concentrations were determined according to the manufacturer's instructions.

CD25 depletion in vitro and in vivo

In vitro purification of $CD4^+$ T cells and depletion of the $CD25^+CD4^+$ subpopulation were performed by cell sorting using a FACSAria (BD Biosciences), which yielded highly pure populations (>98%). In vivo CD25 depletion was performed by injecting anti-CD25 Ab, as described previously (24, 25). Briefly, a hybridoma producing anti-CD25 Ab (PC61; American Type Culture Collection, Manassas, VA) was cultured in serum-free medium (Protein-Free Hybridoma Medium-II from Invitrogen), and the Ab was purified from supernatant by ammonium sulfate precipitation and a PD10 column (GE Healthcare, Buckinghamshire, U.K.). The purified product was quantified using the Bradford assay (Bio-Rad, Hercules, CA) at OD595, and 1 mg was injected i.p. weekly from day 0 for 3 wk. Control rat nonspecific IgG was purchased from Invitrogen.

Quantitative RT-PCR

At day 21 after bone marrow transplantation, $CD25^-CD4^+$ T cells were purified by cell sorting from recipients of WT or IL-$21R^{-/-}$ $CD4^+$ T cells; RNA was isolated (RNeasy, Qiagen, Valencia, CA), reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), and PCR amplified using TaqMan Gene Expression Assay's primer for mouse Foxp3 (Mm00475156) and β-actin (Mm00607939) and an ABI Prism 7700 sequence detection System (Applied Biosystems, Foster City, CA).

Statistical analysis

Kaplan–Meier plots were used to compare survival rates. The log-rank test was used to evaluate p values. Statistical analyses were performed using Stat Mate ver. 6 (ATMS, Tokyo, Japan). The Student t test was used; all error bars in this study represent SD, unless otherwise specified.

Results

Purified $CD4^+$ T cell transplantation and pathological analysis

Decreased GVHD was observed when we transplanted IL-21R–deficient splenocytes compared with WT bulk splenocytes (22). Although we sought to find molecular mechanism(s) for the ameliorated GVHD, no clue was immediately evident from the transplantation experiments (22). Thus, in this study, we used purified $CD4^+$ T cells instead of bulk splenocytes in an effort to augment the differences observed. We used a well-known model of $CD4^+$ T cell-mediated GVHD (26), in which C57BL/6 mice were donors, and C57BL/6-DBA2-F1 mice were recipients. In this model, the difference between WT and IL-$21R^{-/-}$ cells seemed to be greater than in the previous experiments using bulk splenocytes (22). All recipients of WT $CD4^+$ T cells died within 55 d, whereas those receiving IL-$21R^{-/-}$ $CD4^+$ T cells survived during this time period (Fig. 1*A*). Moreover, recipients of IL-$21R^{-/-}$ $CD4^+$ T cells recovered from body weight loss by day 14, but those receiving WT $CD4^+$ T cells did not recover and continued to lose weight (Fig. 1*B*). In recipients of IL-$21R^{-/-}$ $CD4^+$ T cells, pathological analysis showed markedly reduced infiltration into the regions surrounding bile ducts and portal veins and into the interstitial region of small intestine compared with the infiltration observed in recipients of WT $CD4^+$ T cells (Fig. 2, *upper* and *middle panels*). Apoptotic bodies near the surface area of crypts in the small intestine were barely visible in recipients

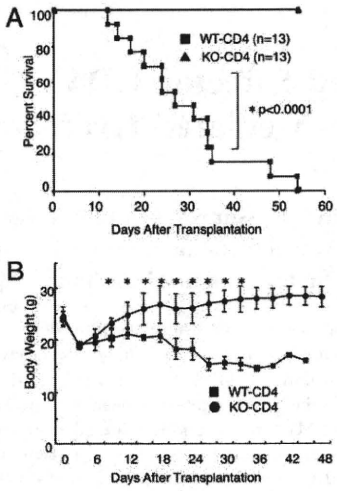

FIGURE 1. A role for IL-21 in $CD4^+$ T cell-mediated GVHD. *A*, Survival of recipients of WT and IL-$21R^{-/-}$ $CD4^+$ T cells. C57BL/6-DBA2-F1 mice were irradiated with 11 Gy and received 5×10^6 IL-$21R^{-/-}$ BM with 5×10^6 WT or IL-$21R^{-/-}$ $CD4^+$ T cells. Shown are combined data from two independent experiments. Thirteen recipients each for WT and IL-$21R^{-/-}$ $CD4^+$ T cells were analyzed. The log-rank test was used to calculate p values. *B*, Body weight after BM transplantation. Statistical significance was assessed with the Student t test.

of IL-$21R^{-/-}$ $CD4^+$ T cells, in contrast to recipients of WT $CD4^+$ T cells, in which apoptotic bodies were evident (Fig. 2, arrowheads in *middle panel*). No significant difference was observed in skin

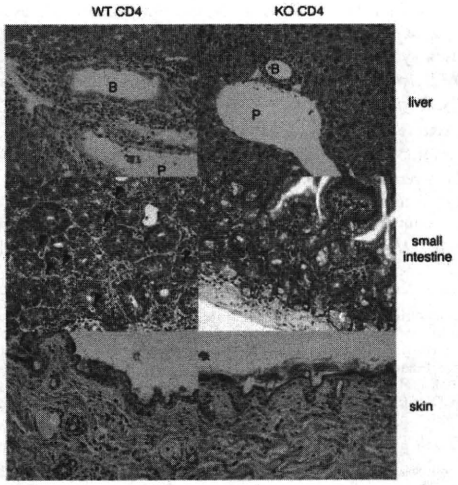

FIGURE 2. Pathological analysis of recipients. Liver, small intestine, and skin were stained with H&E (original magnification ×400). In recipients of WT $CD4^+$ T cells, cell infiltration is evident around the portal vein (P) and the bile duct (B) and into the interstitial region in small intestine. Arrowheads indicate apoptotic bodies near the surface of crypts. These changes were barely visible in recipients of IL-$21R^{-/-}$ $CD4^+$ T cells. Skin did not show any significant difference between recipients of WT and IL-$21R^{-/-}$ $CD4^+$ T cells. Shown is a representative result of six mice analyzed in each group. Only one recipient of IL-$21R^{-/-}$ $CD4^+$ T cells showed apoptotic bodies in the lumens of intestine and infiltration around the bile duct and portal vein, as was observed in the recipients of WT $CD4^+$ T cells.

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pathology among recipients of WT CD4$^+$ and IL-21R$^{-/-}$ CD4$^+$ T cells. These results suggested that IL-21 might be essential for CD4-mediated GVHD, at least in this setting.

Normal cytokine production by splenocytes after transplantation is dependent on IL-21

The above observations suggested that IL-21–mediated donor CD4$^+$ T cell activation was involved in the exacerbation of GVHD. Because we could not find any significant difference in serum cytokine concentrations after transplantation (Supplemental Fig. 1), we assessed T cell differentiation by cytokine production in the presence of cellular stimulation. Interestingly, at days 14 and 21 after transplantation, bulk splenocytes from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells exhibited defective cytokine production, with decreased levels of IFN-γ, TNF-α, and IL-4; in contrast, levels of IL-2, IL-17, and IL-21 were not significantly diminished (Fig. 3, *left panels*). Before transplantation, IL-21R$^{-/-}$ CD4$^+$ T cells did not show any significant defect in IFN-γ, IL-4, or TNF-α production (Fig. 3, *right panels*), suggesting that the defect was acquired after transplantation. This defect in effector T cell function might represent a mechanism for the difference in the development of GVHD by mice receiving WT versus IL-21R$^{-/-}$ CD4$^+$ T cells.

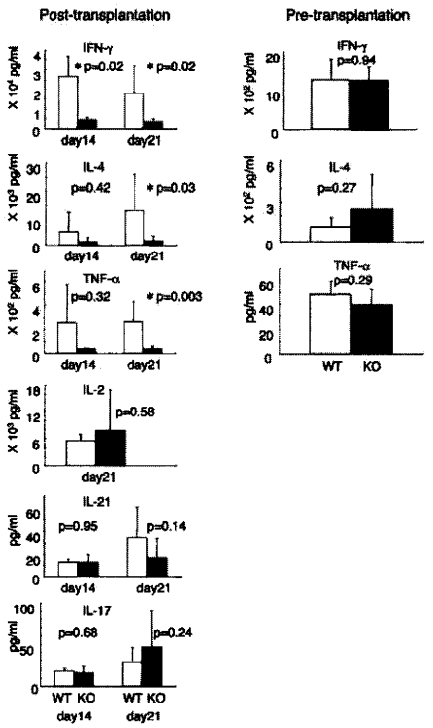

FIGURE 3. Cytokine production by bulk splenocytes before and after CD4$^+$ T cell transplantation. At days 14 and 21 after transplantation, splenocytes (5×10^5) were taken and stimulated with anti-CD3/CD28 Abs for 18 h. Concentrations of cytokines in the supernatants were determined by ELISA. Twelve or 13 recipients of WT CD4$^+$ T cells and 10 recipients of IL-21R$^{-/-}$ CD4$^+$ T cells were analyzed. Prior to transplantation, five WT and eight IL-21R$^{-/-}$ mice were analyzed. At days 14–21 after transplantation, the proportion of donor cells in the spleen was >95%. The Student *t* test was used to calculate *p* values. *Statistical significance ($p < 0.05$).

CD4$^+$ T cells were responsible for the low production of cytokines

To elucidate the basis for diminished cytokine production, we examined the number of donor CD4$^+$ T cells in the spleen at days 14–21 after transplantation. The number of donor H-2K^{d-}CD4$^+$ T cells was significantly lower in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells than in recipients of WT CD4$^+$ T cells (Fig. 4*A*; $p = 0.03$, Welch *t* test; $n = 15$ versus 12), although the ranges overlapped. Because it is thought that donor T cells proliferate in secondary lymphoid organs, such as the spleen, and then infiltrate into target organs (27), the reduced number of CD4$^+$ T cells in the spleen is consistent with the reduced infiltration into the liver and small intestine, as shown above (Fig. 2). To identify the cells responsible for defective cytokine production, we performed intracellular staining and ELISA with purified CD4$^+$ T cells. After anti-CD3/CD28 stimulation, the proportion of IFN-γ$^+$ and TNF-α$^+$ cells in splenic CD4$^+$ T cells was lower in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells than in those receiving WT CD4$^+$ T cells (Fig. 4*B*). Moreover, posttransplantation, the levels

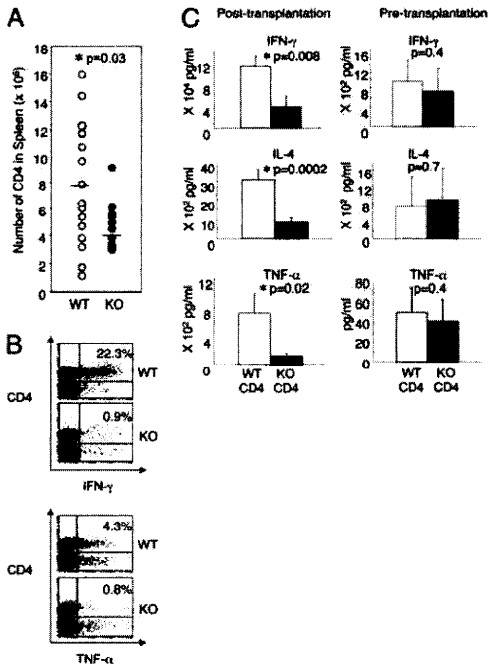

FIGURE 4. Cytokine production by splenic CD4$^+$ T cells before and after transplantation. *A*, Absolute number of donor H-2K^{d-}CD4$^+$ T cells in the spleen. The number of donor CD4$^+$ T cells was determined by multiplying the number of splenocytes by the percentage of H-2K^{d-}CD4$^+$ T cells. Each dot depicts the number of donor CD4$^+$ T cells in a mouse. Horizontal lines indicate the average. Fifteen recipients of WT CD4$^+$ T cells and 12 recipients of IL-21R$^{-/-}$ CD4$^+$ T cells were assessed. *B*, Intracellular staining of splenocytes after anti-CD3/CD28 stimulation. Splenocytes (1×10^6) were stimulated with anti-CD3/CD28 Abs for 5–6 h and stained with anti–IFN-γ or anti–TNF-α Ab in combination with anti-CD4 Ab. A total of three recipients in each group were analyzed, and a representative result is shown. *C*, Cytokine production by CD4$^+$ T cells in vitro. At days 14 or 21 after transplantation, splenic CD4$^+$ T cells (5×10^5) were purified and stimulated with anti-CD3/CD28 Abs for 18 h. Concentrations of cytokines in the supernatants were determined by ELISA. Twelve mice were analyzed in each group after transplantation. Five or six WT and eight or nine IL-21R$^{-/-}$ mice were analyzed before transplantation. *Statistical significance ($p < 0.05$).

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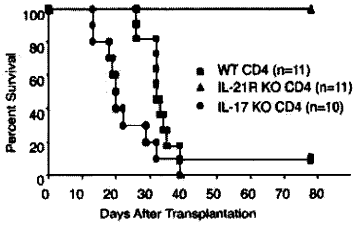

FIGURE 5. IL-17$^{-/-}$ CD4$^+$ T cells induced lethal GVHD. Survival of recipients of WT, IL-21R$^{-/-}$ (IL-21R KO), or IL-17$^{-/-}$ (IL-17 KO) CD4$^+$ T cells. Lethally irradiated (11 Gy) C57BL/6-DBA2-F1 mice were transplanted with 5×10^6 IL-21R KO BM and 5×10^6 WT, IL-21R KO, or IL-17 KO CD4$^+$ T cells. The data represent the combined results of two independent experiments.

of IFN-γ, TNF-α, and IL-4 production were significantly diminished with splenic-purified CD4$^+$ T cells from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells compared with those receiving WT CD4$^+$ T cells (Fig. 4*C*, *left panels*). Before transplantation, IL-21R$^{-/-}$ CD4$^+$ T cells did not show any defect in IFN-γ, TNF-α, and IL-4 production (Fig. 4*C*, *right panels*).

IL-17 production and GVHD induced by IL-17$^{-/-}$ CD4$^+$ T cells

Although IL-21 is not essential for Th17 differentiation, IL-21 can promote it. To evaluate the effect of IL-21$^{-/-}$ CD4$^+$ T cell transplantation on IL-17 production, we measured IL-17 after transplantation. As shown in Fig 3, *bottom left panel*, bulk splenocytes from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells produced comparable amounts of IL-17 at days 14 and 21 after transplantation compared with mice receiving WT CD4$^+$ T cells. Moreover, we found that IL-17$^{-/-}$ CD4$^+$ T cells induced lethal GVHD analogous to WT CD4$^+$ T cells (if anything, death occurred earlier), suggesting that IL-17 is dispensable for this process, in contrast to the essential role of IL-21, as reflected by the survival of mice receiving IL-21R$^{-/-}$ CD4$^+$ T cells (Fig. 5).

Regulatory T cell number in spleen

We next determined the serum concentration of the major immunosuppressive cytokine, TGF-β1, at days 6–21 after transplantation. We found an increase in TGF-β1 only after transplantation (Fig. 6*A*; $p = 0.0003$ at day 14; $p = 0.01$ at day 21, Student t test). In splenocytes from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells, the production of TGF-β1 and IL-10 by in vitro T cell stimulation was not upregulated; in fact, it tended to be diminished (Supplemental Fig. 2), suggesting that the increase in serum TGF-β1 might be due to cells other than T cells. Because naive T cells can differentiate into regulatory T (Treg) cells in the presence of TGF-β1 (28), and it was reported that IL-21$^{-/-}$ T cells were predisposed to differentiate into Treg cells (8), we also investigated whether more Treg cells were induced in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells. The proportion

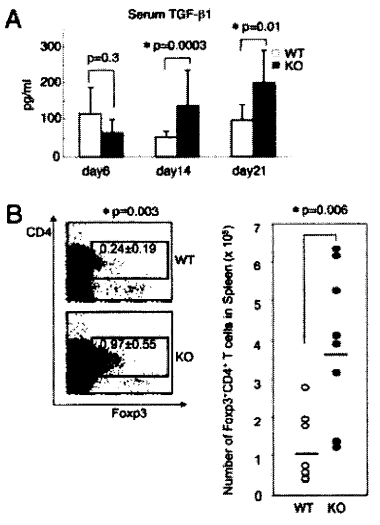

FIGURE 6. Increase in splenic Treg cells. *A*, Upregulation of serum TGF-β1. Serum TGF-β1 concentrations at the indicated day after transplantation were determined by ELISA. Three samples from recipients of WT CD4$^+$ T cells and 4 samples from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells at day 6, 23 samples from recipients of WT CD4$^+$ T cells and 25 samples from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells at day 14, and 8 samples from recipients of WT CD4$^+$ T cells and 7 samples from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells at day 21 were analyzed. *Statistical significance ($p < 0.05$). *B*, The percentage and absolute number of splenic Foxp3$^+$CD4$^+$ regulatory T cells at day 14 after transplantation. The left panel shows a representative flow cytometric result from eight or nine similar samples. The right panel indicates the number of all samples; the averages are indicated by the horizontal lines.

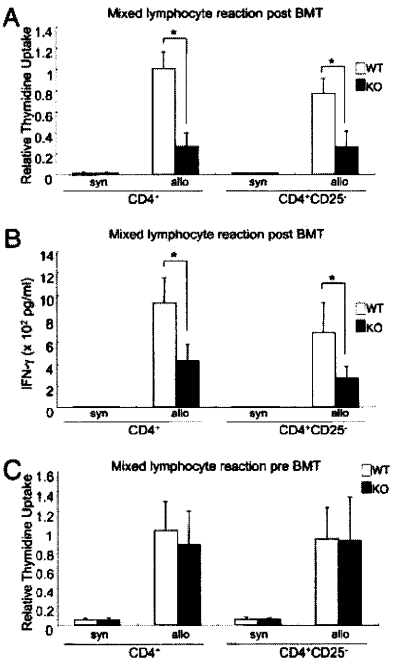

FIGURE 7. An impaired CD4 alloreaction is not dependent on CD25$^+$ CD4$^+$ T cells. CD4 alloreaction in vitro was impaired after transplantation, and this impairment was not restored by CD25$^+$ T cell depletion. *A*, At day 14 after transplantation, 1×10^5 sorter-purified splenic CD4$^+$ or CD25$^-$ CD4$^+$ T cells (>98% purity) were cultured with 4×10^5 irradiated allogeneic C57BL/6-DBA2-F1 splenocytes for 4 d. The cells were pulsed with 1 μCi of [^{3}H]thymidine for the last 24 h. Relative thymidine uptake to the value of WT CD4$^+$ T cells is depicted. *B*, Culture was the same as in *A*, but IFN-γ concentrations in the supernatants were determined by ELISA. *C*, Sorter-purified splenic CD4$^+$ or CD25$^-$CD4$^+$ cells from nontransplanted mice were cultured with irradiated allogeneic C57BL/6-DBA2-F1 splenocytes. Relative thymidine uptake to the number of WT CD4$^+$ T cells is depicted. *$p < 0.05$.

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of splenic Foxp3$^+$CD4$^+$ Treg phenotype cells in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells was higher than in recipients of WT CD4$^+$ T cells, but the total percentage was still only ~1% (Fig. 6*B*, *left panel*). The absolute number was ~4-fold higher, but the actual number was only ~4 × 10^5 of the total number of splenocytes (~4 × 10^7) (Fig. 6*B*, *right panel*). In contrast to posttransplantation, pretransplantation splenocytes from IL-21R$^{-/-}$ mice did not show an increase in Foxp3$^+$CD4$^+$ T cells compared with cells from WT mice (Supplemental Fig. 3), suggesting that the increased Treg cell after transplantation was an induced Treg cell during GVHD reaction. For that reason, we did not deplete CD25$^+$ cells prior to transplantation.

CD25 depletion did not restore the suppressed alloreaction in vitro and did not exacerbate the ameliorated GVHD

To investigate the importance of Treg cells in diminishing GVHD, we performed an MLR, which corresponds to alloreaction in vitro, with or without CD25$^+$CD4$^+$ T cells. Because Foxp3 is an intracellular protein, and Foxp3 staining cannot be used to purify or deplete Treg cells, anti-CD25 Ab is widely used for this purpose (9, 29–32). The impaired MLR of IL-21R$^{-/-}$ CD4$^+$ T cells after transplantation was not restored by CD25 depletion (Fig. 7*A*), nor was the impaired IFN-γ production by IL-21R$^{-/-}$CD4$^+$ T cells in an MLR (Fig. 7*B*). Moreover, analogous to cytokine production by anti-CD3/CD28 stimulation (Fig. 3), IL-21R$^{-/-}$ CD4$^+$ T cells before transplantation were not defective for alloreaction (Fig. 7*C*).

Consistent with the in vitro experiments above, CD25$^+$ depletion in vivo did not alter the severity of GVHD in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells, without altering the body weight loss and survival (Fig. 8*A*, 8*B*). In contrast, the severity of GVHD in recipients of WT CD4$^+$ T cells seemed to be slightly diminished by CD25$^+$ depletion (Fig. 8*A*, 8*B*). In this condition, as previously reported (30), the depletion efficacy of CD25$^+$CD4$^+$ T cells was >95% and that of Foxp3$^+$CD4$^+$ T cells was ≅50% (Fig. 8*C*, *upper* and *lower panels*). Interestingly, Foxp3 expression was higher in CD25$^-$CD4$^+$ T cells from recipients of IL-21R$^{-/-}$ CD4$^+$ T cells than from recipients of WT CD4$^+$ T cells (Fig. 8*D*). Together with the results in vitro (Fig. 7), this suggests a relationship between the unresponsiveness of CD25$^-$CD4$^+$ T cells and greater expression of Foxp3.

Discussion

In this article, we reported evidence indicating that IL-21 is critical for the pathogenesis of CD4$^+$ T cell-mediated GVHD, at least in part because of its effects on CD4 differentiation. In this study, we focused on CD4$^+$ T cell-mediated GVHD; a role for IL-21 in CD8$^+$ T cell-mediated GVHD remains to be investigated.

We found a profound defect in T cell effector function only after transplantation, although serum cytokine concentrations showed no obvious difference. According to these results, T cell differentiation into Th1 and Th2 cells seemed to be altered in the absence of IL-21 during GVHD. Cytokines are believed to have positive and negative roles in GVHD. For example, although T cells from IFN-γ–deficient mice resulted in more severe GVHD (33–35), T cells from Stat4 (Th1)-deficient mice resulted in less severe GVHD than did T cells from WT mice with less severe colitis (36). In contrast to IFN-γ$^{-/-}$ T cells, T cells from IL-4–deficient mice induced less severe GVHD (34); analogously, T cells from Stat6 (Th2)-deficient mice induced less severe GVHD than did those from WT mice (36). T cells from TNF-α–deficient mice developed less severe GVHD, with less severe colitis (37). Our data suggest a strong correlation between the defect in effector function in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells and the attenuated phenotype of GVHD, indicating a role for IL-21 in this process.

IL-21, as well as IL-6, induces Th17 differentiation in the presence of TGF-β, suggesting a possible involvement of IL-17 in

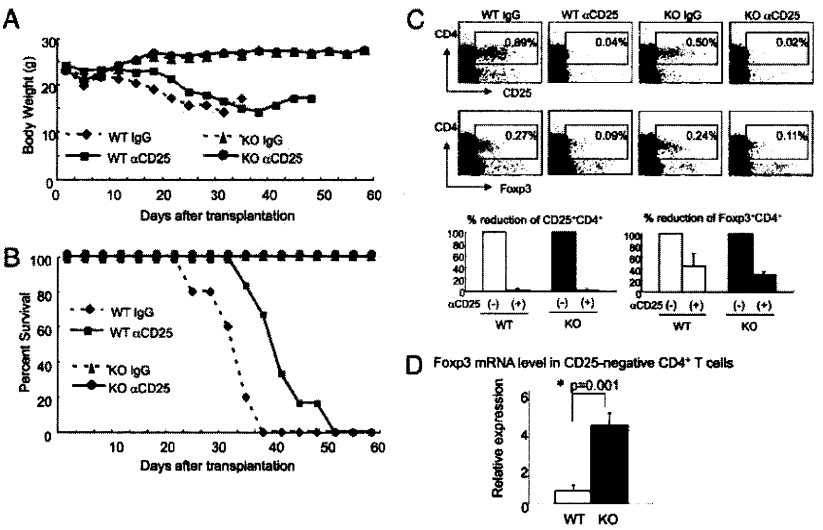

FIGURE 8. The ameliorated GVHD induced by IL-21R$^{-/-}$ CD4$^+$ T cells is not dependent on CD25$^+$CD4$^+$ T cells. The ameliorated GVHD induced by IL-21R$^{-/-}$ CD4$^+$ T cells was not exacerbated by depletion of CD25$^+$CD4$^+$ T cells. Body weight (*A*) and survival (*B*) of recipients are shown. Comparisons of recipients of WT CD4$^+$ T cells and IL-21R$^{-/-}$ CD4$^+$ T cells and additional comparisons with and without anti-CD25 Ab treatment were performed. Nonspecific rat IgG was used as the control Ab. *C*, Splenic CD25$^+$CD4$^+$ T cells and splenic Foxp3$^+$CD4$^+$ T cells at day 14 after transplantation with or without anti-CD25 Ab treatment were analyzed by flow cytometry (*upper two rows*). The lower panels indicate the mean reduction in the percentage of CD25$^+$CD4$^+$ and Foxp3$^+$CD4$^+$ cells from three similar results. *D*, Foxp3 mRNA level in CD25$^-$CD4$^+$ T cells at day 21 after transplantation. Cell sorter-purified CD25$^-$CD4$^+$ T cells were subjected to mRNA purification, reverse-transcriptase treatment, and TaqMan quantitative PCR. Relative value to β-actin is denoted.

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the phenotype we observed. However, our results with IL-17$^{-/-}$ CD4$^+$ T cells demonstrated that IL-17 was dispensable for CD4$^+$ T cell-mediated GVHD, indicating that the attenuated GVHD in recipients of IL-21R$^{-/-}$ CD4$^+$ T cells was not due to an IL-17–related defect. During the preparation of this manuscript, a role for IL-17 in GVHD was reported (38–40). These reports varied, but one suggested that the lack of IL-17 promotes GVHD (38). Another report suggested that IL-17$^{-/-}$ CD4$^+$ T cells can ameliorate GVHD only at the early stages, which suggested a promoting effect for IL-17 at an early stage of GVHD (39). The third report suggested that ex vivo-differentiated Th17 cells induced skin and lung GVHD (40). Thus, the role of IL-17 may be complex and dependent on the specific experimental conditions.

Because there are reciprocal relationships between Th1/Th2 and Treg cell differentiation (41–43) and between IL-21 and Treg cell differentiation (8), we investigated the level of Treg cells in the spleens of recipients. Foxp3$^+$CD4$^+$ T cells were increased in percentage and absolute number but still represented only ~1% of splenocytes. Regarding the relationship between the defective effector T cell function and the increased number of Treg cells, it is possible that increased Treg cells suppress functional effector T cells. Alternatively, it is possible that effector differentiation itself is defective, and the resulting effector T cells cannot respond to alloantigen, analogous to the situation in T cell anergy, and that the increased Treg cell number is also a result of a dysregulated differentiation. Our results might be more consistent with the latter possibility, given that Treg cell depletion by anti-CD25 treatment did not alter the results in vitro and in vivo, although the efficiency of depletion of Foxp3$^+$CD4$^+$ T cells in vivo was incomplete. It is also conceivable that the upregulation of Foxp3 in CD25$^-$CD4$^+$ T cells (which would not be removed by CD25$^+$ depletion) in the absence of IL-21 signaling might result in unresponsiveness or poor responsiveness of effector T cells and that more than one mechanism can contribute to the attenuated GVHD.

Disclosures

K. Ozaki and W.J.L. are inventors on patents and patent applications related to IL-21.

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ORIGINAL CONTRIBUTION

Hematopoietic Stem Cell Transplantation
A Global Perspective

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Context Hematopoietic stem cell transplantation (HSCT) requires significant infrastructure. Little is known about HSCT use and the factors associated with it on a global level.

Objectives To determine current use of HSCT to assess differences in its application and to explore associations of macroeconomic factors with transplant rates on a global level.

Design, Setting, and Patients Retrospective survey study of patients receiving allogeneic and autologous HSCTs for 2006 collected by 1327 centers in 71 participating countries of the Worldwide Network for Blood and Marrow Transplantation. The regional areas used herein are (1) the Americas (the corresponding World Health Organization regions are North and South America); (2) Asia (Southeast Asia and the Western Pacific Region, which includes Australia and New Zealand); (3) Europe (includes Turkey and Israel); and (4) the Eastern Mediterranean and Africa.

Main Outcome Measures Transplant rates (number of HSCTs per 10 million inhabitants) by indication, donor type, and country; description of main differences in HSCT use; and macroeconomic factors of reporting countries associated with HSCT rates.

Results There were 50 417 first HSCTs; 21 516 allogeneic (43%) and 28 901 autologous (57%). The median HSCT rates varied between regions and countries from 48.5 (range, 2.5-505.4) in the Americas, 184 (range, 0.6-488.5) in Asia, 268.9 (range, 5.7-792.1) in Europe, and 47.7 (range, 2.8-95.3) in the Eastern Mediterranean and Africa. No HSCTs were performed in countries with less than 300 000 inhabitants, smaller than 960 km^2, or having less than US $680 gross national income per capita. Use of allogeneic or autologous HSCT, unrelated or family donors for allogeneic HSCT, and proportions of disease indications varied significantly between countries and regions. In linear regression analyses, government health care expenditures (r^2=77.33), HSCT team density (indicates the number of transplant teams per 1 million inhabitants; r^2=76.28), human development index (r^2=74.36), and gross national income per capita (r^2=74.04) showed the highest associations with HSCT rates.

Conclusion Hematopoietic stem cell transplantation is used for a broad spectrum of indications worldwide, but most frequently in countries with higher gross national incomes, higher governmental health care expenditures, and higher team densities.

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HEMATOPOIETIC STEM CELL transplantation (HSCT) has become the standard of care for many patients with defined congenital or acquired disorders of the hematopoietic system or with chemosensitive, radiosensitive, or immunosensitive malignancies.[1-3] Over the last 2 decades, HSCT has seen rapid expansion in use and a constant evolution in its technology. Novel indications are currently under evaluation.[4,5] Bone marrow is supplemented as a stem cell source by peripheral blood or cord blood. More than 14 million typed volunteer donors or cord blood units from the many registries worldwide provide stem cells for patients without family donors. Novel conditioning regimens with lower intensity have expanded the use of HSCT to older patients and to those with comorbidities.[6-9]

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Still, HSCT remains associated with significant morbidity and mortality and represents one example of high-cost, highly specialized medicine. It requires significant infrastructure and a network of specialists from all fields of medicine. Hence, information on indications, use of specific technologies, and trends in the application of HSCT is essential for correct patient counseling and for health care agencies to prepare the necessary infrastructure and to avoid planning errors.[10-13] In addition, HSCT is no longer limited to countries with abundant resources. For selected indications, HSCT might represent the most cost-effective therapy in some countries.[14] An assessment of global HSCT activity is warranted.

In view of the increasing numbers of transplant teams and HSCTs worldwide and the increasing awareness of the need for a global perspective for all cell, tissue, and organ transplants by the World Health Organization,[15] the recently founded Worldwide Network for Blood and Marrow Transplantation decided to collect standardized HSCT activity data on a global level. Results of the first worldwide HSCT survey are presented herein.

METHODS

Study Design

This is a retrospective survey among all HSCT teams known to the investigators, which was organized by the Worldwide Network for Blood and Marrow Transplantation through established international and regional organizations. The study was approved by the ethics committee of the University of Basel; and the need for informed consent of patients was waved because no individualized data was transferred to the investigators.

The main outcome measures were the determination of transplant rates (number of HSCTs per 10 million inhabitants) by indication, donor type, and country on a global level. Secondary outcomes were the description of the main differences in HSCT use and the key macroeconomic factors of the reporting countries and regions associated with their transplant rates.

Participating Groups, Continents, Countries, and Teams

There were 1327 teams in 71 reporting countries over 5 continents (see eTable at http://www.jama.com) that provided information on numbers of HSCT for 2006 by indication and donor type (TABLE 1).[16] They were subdivided into 4 regions: (1) the Americas (the corresponding World Health Organization regions are North and South America), (2) Asia (Southeast Asia and the Western Pacific Region, which includes Australia and New Zealand), (3) Europe (which includes Tur-

Table 1. Hematopoietic Stem Cell Transplants Worldwide in 2006[a]

	Allogeneic Donor				
	Family (n = 11 928)	Unrelated (n = 9588)	Total (n = 21 516)	Autologous Donor (n = 28 901)	Total (N = 50 417)
Leukemia	8122 (68.1)	7088 (73.9)	15 210 (70.7)	1839 (6.4)	17 049 (33.8)
Acute myeloid leukemia	3907 (48.1)	3119 (44.0)	7026 (46.2)	1372 (74.6)	8398 (49.3)
Acute lymphoblastic leukemia	1799 (22.1)	1850 (26.1)	3649 (24.0)	216 (11.7)	3865 (22.7)
Myelodysplastic, myeloproliferative syndromes	1151 (14.2)	1248 (17.6)	2399 (15.8)	60 (3.3)	2459 (14.4)
Chronic myeloid leukemia	877 (10.8)	519 (7.3)	1396 (9.2)	14 (1.0)	1410 (8.3)
Chronic lymphocytic leukemia	336 (4.1)	269 (3.8)	605 (4.0)	175 (9.5)	780 (4.6)
Other leukemia	52 (1.0)	83 (1.2)	135 (1.0)	2 (<1.0)	137 (1.0)
Lymphoproliferative disorders	2088 (17.5)	1414 (14.7)	3502 (16.3)	23 990 (83.0)	27 492 (54.5)
Plasma cell disorders	546 (26.1)	287 (20.3)	833 (23.8)	11 877 (49.5)	12 710 (46.2)
Hodgkin disease	270 (12.9)	235 (16.6)	505 (14.4)	3275 (13.7)	3780 (13.7)
Non-Hodgkin lymphoma	1109 (53.1)	708 (50.1)	1817 (51.9)	7943 (33.1)	9760 (35.5)
Other lymphoma (type unknown)	163 (8.0)	184 (13.0)	347 (10.0)	895 (4.0)	1242 (5.0)
Solid tumors	113 (1.0)	40 (<1.0)	153 (<1.0)	2772 (9.6)	2925 (5.8)
Neuroblastoma	22 (19.5)	8 (20.0)	30 (19.6)	615 (22.2)	645 (22.1)
Germinal cancer	3 (3.0)	2 (5.0)	5 (3.3)	518 (18.7)	523 (17.9)
Breast cancer	13 (11.5)	4 (5.0)	17 (11.1)	273 (9.8)	290 (10.0)
Ewing sarcoma	17 (15.0)	6 (20.0)	23 (15.0)	176 (6.3)	199 (6.8)
Other	58 (51.3)	20 (50.0)	78 (51.0)	1190 (42.9)	1268 (43.4)
Nonmalignant disorders	1512 (12.7)	884 (9.0)	2396 (11.1)	197 (1.0)	2593 (5.1)
Bone marrow failures	879 (58.1)	457 (52.0)	1336 (55.8)	0	1336 (51.5)
Hemoglobinopathies	348 (23.0)	54 (6.1)	402 (16.8)	3 (1.5)	405 (15.6)
Immune deficiencies	216 (14.3)	241 (27.3)	457 (19.1)	3 (1.5)	460 (17.7)
Inherited diseases of metabolism	63 (4.0)	122 (13.8)	185 (7.7)	2 (1.0)	187 (7.2)
Autoimmune disorders	6 (<1.0)	10 (1.1)	16 (1.0)	189 (96.0)	205 (8.0)
Other	93 (1.0)	162 (2.0)	255 (1.2)	103 (<1.0)	358 (1.0)

[a]Values are expressed as number (column percentage of total and within subgroup). Percentages may not equal 100% due to rounding.

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key and Israel), and (4) the Eastern Mediterranean and Africa.

Data were provided by the Asian Pacific Blood and Marrow Transplant Group, the Australian Bone Marrow Transplant Recipient Registry, the Canadian Blood and Marrow Transplant Group, the Center for International Blood and Marrow Transplantation, the Sociedade Brasileira de Transplante de Medula Ossea, the Eastern Mediterranean Blood and Marrow Transplant Group, and the European Group for Blood and Marrow Transplantation (see eTable at http://www.jama.com).[17-20]

Collection System and Data Validation

Data were obtained from mandatory reporting systems of initial transplant data (Australian Bone Marrow Transplant Recipient Registry, Canadian Blood and Marrow Transplant Group, and Center for International Blood and Marrow Transplantation) or collected on separate survey data forms from individual centers or national registries (Asian Pacific Blood and Marrow Transplant Group, European Group for Blood and Marrow Transplantation, Eastern Mediterranean Blood and Marrow Transplant Group, and Sociedade Brasileira de Transplante de Medula Ossea).

Data were validated by several independent methods. The data were first confirmed by the reporting team, which received a computer printout of the entered data. Selective comparison also was used with Med-A data sets in the European Group for Blood and Marrow Transplantation Promise data system or by cross-checking with national registries. Onsite visits of selected teams were part of the quality-control program within the Center for International Blood and Marrow Transplantation and the European Group for Blood and Marrow Transplantation.

Based on quality controls and contacts with regulatory agencies or national offices, response rates of allogeneic HSCT was greater than 95% in Australia, Brazil, Canada, Europe, Japan, Korea, Malaysia, New Zealand, Taiwan, and the United States. No formal response rate can be evaluated for the other participating countries; there is no formal regulatory framework for cross-confirmation. Concerning autologous HSCT, the response rate in Europe was greater than 90% and it can be estimated to be between 80% and 90% for Australia, Brazil, Canada, Europe, Japan, Korea, Malaysia, New Zealand, Taiwan, and the United States. For autologous HSCT, no formal framework exists to capture nonreporting teams and to validate response rates with accuracy.

Definitions

This Worldwide Network for Blood and Marrow Transplantation survey focused on the numbers of patients treated for the first time with HSCT in 2006. Information on additional transplants (eg, retransplants or multiple HSCTs[21]) was not included.

Transplant rates were computed as the number of HSCTs per 10 million inhabitants.[21] Transplant rates refer to the number of transplants in a given country compared with its own population, without adjustments for patients who cross borders and receive a HSCT in a foreign country. Population data were obtained from the US census office.

Team density refers to the number of transplant teams per 1 million inhabitants.[22] The definition of a team followed the principles of the Foundation for the Accreditation of Cellular Therapy and the Joint Accreditation Committee of the International Society for Cellular Therapy and the European Group for Blood and Marrow Transplantation.

Transplant rates within the reporting participating countries were compared with a range of macroeconomic health care indicators: gross national income per capita; total health care expenditures; governmental health care expenditures; adult, infant and maternal mortality rate; number of hospital beds per capita; cesarean delivery rates; human developmental index, which is a composite index reflecting the developmental status of all countries in the world in a scale from 0 to 1.0; and team density, which indicates the number of transplant teams per 1 million inhabitants. Data were obtained from the World Bank, the World Health Organization, and the United Nations. Data from 2006 were used for all comparisons whenever possible.

Statistical Analysis

The association of the macroeconomic factors with HSCT rates was estimated by single linear and multiple linear regression analysis, using the least squares method. The linear relationship, positive or negative, between the macroeconomic factors and HSCT rates after transformation was measured using the t statistic; a level of 5% was considered significant. The goodness of fit was measured using the coefficient of determination (r^2). For the single and multiple linear regression analyses, the dependent variables were transformed to point out the linear associations. In the multiple regression analyses, all factors were assessed for their multicollinearity. Taiwan and Hong Kong were excluded from the multiple economic comparisons because of missing information on governmental health care expenditures. Cesarean delivery rates were included in the single linear analyses but not the multiple regression analyses, because data from too many countries were missing.

The t test was used to evaluate if the 4 world regions had a significant difference in the relative proportion of main indications and donor type (allogeneic vs autologous, unrelated vs family donors); $P = .05$ was considered significant. All statistical analyses were performed with EViews version 5.1 (Quantitative Micro Software, Irvine, California).

RESULTS

A total 50 417 first HSCTs were reported for 2006; 21 516 allogeneic (43%) and 28 901 autologous (57%) (Table 1). The main indications were lymphoproliferative disorders (27 492

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patients [54.5%]; 3502 allogeneic [13%] and 23 990 autologous [87%]); leukemias (17 049 patients [33.8%]; 15 210 allogeneic [89%] and 1839 autologous [11%]); solid tumors (2925 patients [5.8%]; 153 allogeneic [5%] and 2772 autologous [95%]); nonmalignant disorders (2593 patients [5.1%]; 2396 allogeneic [92%] and 197 autologous [8%]), and other nonspecified disorders (358 patients; 1%).

The most frequent malignant disease for an allogeneic HSCT was acute myeloid leukemia (n = 7026; 33%), the most frequent nonmalignant disease was bone marrow failure syndrome (n = 1336; 6%), and the most frequent indication for an autologous HSCT was a plasma cell disorder (n = 11 877; 41%).

Most of the 50 417 HSCTs were performed in Europe with 24 216 (48%) (median [range], 255 [6-4619] per country) followed by the Americas with 17 875 (36%) (median [range], 61 [8-15 082] per country), Asia with 7096 (14%) (median [range], 139 [5-3823] per country), and the Eastern Mediterranean and Africa with 1230 (2%) (median [range], 63 [10-360] per country). The absolute numbers of HSCTs in the participating countries ranged from 15 082 in the United States to 5 in Vietnam.

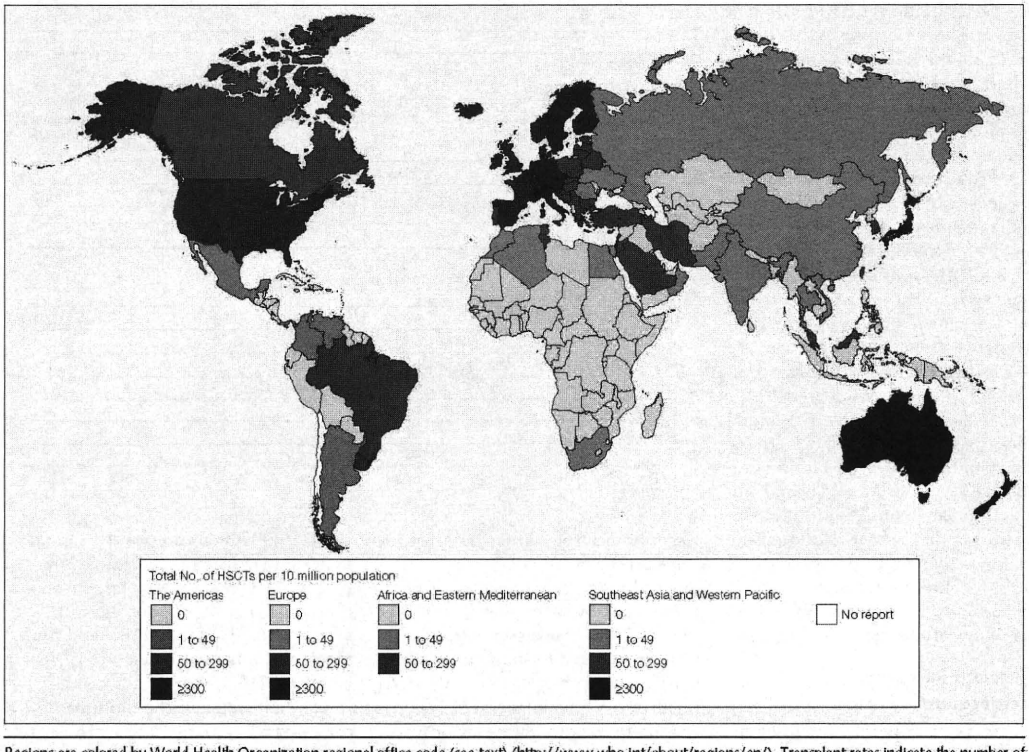
Transplant Rates in 2006

The median HSCT rates varied between the continental regions and between participating countries from 48.5 (range, 2.5-505.4) in the Americas, 184 (range, 0.6-488.5) in Asia, 268.9 (range, 5.7-792.1) in Europe, and 47.7 (range, 2.8-95.3) in the Eastern Mediterranean and Africa (FIGURE 1). Transplant rates for allogeneic HSCT ranged from 434.9 in Israel to 0.2 in Vietnam. Transplant rates for autologous HSCT ranged from 500 in Iceland to 0.3 in Mexico.

Regional Differences in Donor Type and Main Indications

Overall, there were more autologous HSCTs (n = 28 901; 57%) than allogeneic HSCTs (n = 21 516; 43%) (TABLE 2). Most of the autologous HSCTs occurred in the Americas and Europe. In other regions, allogeneic HSCTs were more common (Asia:

Figure 1. Global Distribution of Hematopoietic Stem Cell Transplantations (HSCTs) in 2006



Regions are colored by World Health Organization regional office code (see text) (http://www.who.int/about/regions/en/). Transplant rates indicate the number of first HSCTs per 10 million inhabitants in 2006 and are allogeneic and autologous by continental region.

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Table 2. Allogeneic and Autologous Hematopoietic Stem Cell Transplants by Region[a]

	Americas (n = 17 875)	Asia (n = 7096)	Europe (n = 24 216)	Eastern Mediterranean and Africa (n = 1230)	Total (N = 50 417)
Allogeneic donor	7527 (42.1)	4058 (57.2)	9128 (37.7)	803 (65.3)	21 516 (42.7)
Relationship					
Family	4277 (57.0)	1948 (48.0)	4906 (53.7)	797 (99.3)	11 928 (55.4)
Unrelated	3250 (43.2)	2110 (52.0)	4222 (46.3)	6 (<1.0)	9588 (44.6)
Leukemia	5156 (68.5)	3119 (76.9)	6443 (70.6)	492 (61.3)	15 210 (70.7)
Lymphoproliferative disorders	1466 (19.5)	429 (10.6)	1579 (17.3)	28 (3.5)	3502 (16.3)
Solid tumors	32 (<1.0)	37 (1.0)	83 (1.0)	1 (<1.0)	153 (<1.0)
Nonmalignant disorders	755 (10.0)	418 (10.3)	946 (10.4)	277 (34.5)	2396 (11.1)
Other	118 (2.0)	55 (1.4)	77 (1.0)	5 (<1.0)	255 (1.2)
Autologous donor	10 348 (57.9)	3038 (42.8)	15 088 (62.3)	427 (34.7)	28 901 (57.3)
Leukemia	443 (4.3)	202 (6.6)	1136 (7.5)	58 (13.6)	1839 (6.4)
Lymphoproliferative disorders	8936 (86.4)	2380 (78.3)	12 336 (81.8)	338 (79.2)	23 990 (83.0)
Solid tumors	895 (8.6)	389 (12.8)	1459 (9.7)	29 (6.8)	2772 (9.6)
Nonmalignant disorders	49 (<1.0)	23 (1.0)	123 (1.0)	2 (<1.0)	197 (1.0)
Other	25 (<1.0)	44 (1.4)	34 (<1.0)	0	103 (<1.0)

[a]Values are expressed as number (column percentage of total and within subgroup). Percentages may not equal 100% due to rounding.

57.2%; the Eastern Mediterranean and Africa: 65.3%). The differences in the prevalences of allogeneic HSCTs and the proportions of unrelated donor HSCTs are presented in TABLE 3. The proportion of unrelated donor HSCT was highest in Asia (52%), but it was negligible in the Eastern Mediterranean and Africa (1%).

Leukemia was the main indication for allogeneic HSCT globally (71% overall; the Americas, 68%; Asia, 77%; Europe, 71%; Eastern Mediterranean and Africa, 61%). Nonmalignant diseases comprised about 11% in the Americas, Asia, and Europe and 34% in the Eastern Mediterranean and Africa (see Table 2). Lymphoma was the most common indication for autologous HSCT (79%) in the Eastern Mediterranean and Africa. Plasma cell disorders were the most common indications for autologous HSCT in the Americas and Europe. Compared with Asia, among individuals in the Eastern Mediterranean and Africa there were more allogeneic HSCTs for chronic myelogenous leukemia (28% vs 7%, respectively) and hemoglobinopathies (26% vs 11%).

Table 3. Allogeneic and Unrelated Donor Hematopoietic Stem Cell Transplantations (HSCTs)

Comparison	Allogeneic HSCT, %	Test Statistic	Critical Value at 5% Level	Degrees of Freedom	*P* Value
Asia	57				
vs Americas	42	3.34	2.16	13	.005
vs Europe	38	4.24	2.20	12	.001
Americas	42				
vs Eastern Mediterranean and Africa	65	−4.21	2.23	11	.002
vs Europe	38	1.66	2.10	18	.11
Europe	38				
vs Eastern Mediterranean and Africa	65	−4.96	2.23	10	.001
	Family vs Unrelated Donor				
Eastern Mediterranean and Africa	99				
vs Americas	57	−10.00	2.23	11	<.001
vs Asia	48	−8.40	2.20	12	<.001
vs Europe	54	−13.31	2.02	17	<.001
Americas	57				
vs Asia	48	−0.92	2.11	17	.37
vs Europe	54	0.15	2.11	17	.88
Asia	48				
vs Europe	54	−0.885	2.13	15	.39

Transplant Rates and Macroeconomic Factors

No HSCTs were performed in countries with less than 300 000 inhabitants, smaller than 960 km^2, or having less than US $680 gross national income per capita. All macroeconomic factors had a significant positive or negative association with transplant rates in single regression analyses with a widely variable explanatory content: gross national income per capita (r^2=74.04); total health care expenditures (r^2=73.41); governmental health care expenditures (r^2=77.33) (FIGURE 2A and interactive graphs at at http://www.jama.com); adult (r^2=49.03), infant (r^2=66.31), and maternal (r^2=63.21) mortality rates; hospital beds (r^2=32.04); cesarean section rates (r^2=30.56); and team density (r^2=76.28) (Figure 2B); and human developmental index (r^2=74.36) (Figure 2C).

The first factor in the multiple linear regression analysis, government health care expenditure (GOV), explained 77.33% of the variance of the

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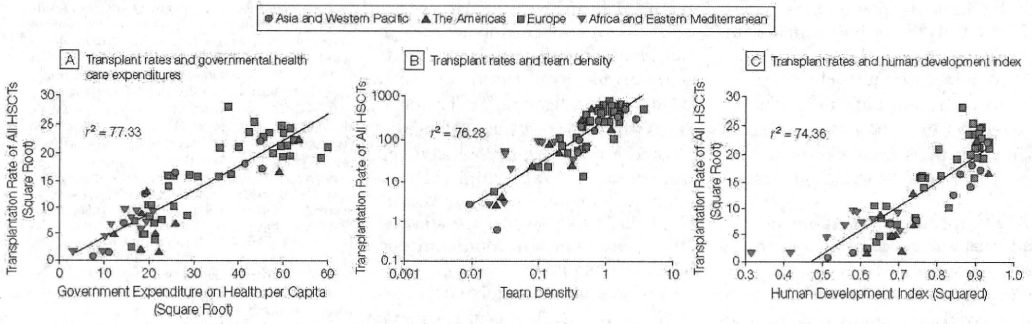
Figure 2. Macroeconomic Factors and Transplant Rates



Transplant rates indicate the number of first hematopoietic stem cell transplantations (HSCTs) per 10 million inhabitants. Team density indicates the number of transplant teams per 1 million inhabitants. See "Methods" section for explanation of the human development index. Interactive graphs are available at http://www.jama.com.

HSCT rates. The second factor, team density (TD), increased R^2 to 79.83%, and the third factor, gross national income (GNI) per capita, added another 4.41% of explanation. All other factors, including the human development index, became insignificant, mainly due to multicollinearity with gross national income per capita, meaning that several factors did correlate highly with each other. Therefore, the equation of the multiple regressions was

$$\sqrt{TR} = c_1\sqrt{GOV} + c_2 \ln(TD) + c_3 \ln(GNI) + \varepsilon$$

Hence, the combined explanatory content was $R^2 = 84.24$.

COMMENT

This first report by the Worldwide Network for Blood and Marrow Transplantation documents the current state of HSCT on a global level. It describes the achievements, illustrates the major differences, and points to the key needs. Transplant activity is concentrated in countries with higher governmental health care expenditures, higher gross national income per capita, and higher team density. Hence, availability of resources, governmental support, and access to a transplant center are the key factors related to regional HSCT activity. However, disease prevalence can differ between regions and could contribute to differences in HSCT rates; those data were not included in this report.

The close link of HSCT rates with gross national income per capita was recognized many years ago; HSCT is an expensive procedure with a substantial investment for a single patient.[21] No HSCTs were performed in countries with less than US $700 gross national income per capita. However, gross national income per capita explained only parts of the variations. Therefore, we were specifically interested in other macroeconomic factors associated with HSCT rates. These factors were chosen with intention. They were either directly linked to availability of resources (gross national income per capita, health care expenditures), to governmental support (governmental health care expenditures), or to the overall infrastructure in a country (human development index). Others reflect quality measures of the health care system (mortality rates) or indicate potential overuse of the health care system (hospital beds, cesarean delivery). Of all macroeconomic factors, this study identified governmental health care expenditures as the most closely associated factor with HSCT rates.

Our study could not assess the role of the health care system in the participating countries because there is no globally accepted definition available. Definitive explanations cannot be given, but some assumptions can be made. The cost-effectiveness of HSCT compared with conventional treatment has at least recently been discussed for patients with chronic myeloid leukemia in middle-income countries.[14,23] Transplant rates were strongly associated with team density. There was no indication for saturation in this association. Hence, a minimum number of transplant teams per inhabitants must be available so that patients have sufficient access. It does not appear that transplant teams overuse their infrastructure.[22,24] None of the other traditional health care indicators or the composite human development index provided a higher explanatory content or added information in the multiple regression analyses.

There were significant differences between the regions concerning indications and donor type, with fewer autologous HSCTs in Asia and the Eastern Mediterranean and Africa than in the Americas and Europe. There were more unrelated donors for HSCTs in the Americas, Asia, and Europe than in the Eastern Mediterranean and Africa; the highest proportion of unrelated donors for HSCTs was in Japan. There also were more HLA identical sibling donor HSCTs for congenital disorders or

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for aplastic anemia in countries with limited resources. A matched sibling donor HSCT might represent the most efficient way of therapy for a patient with aplastic anemia, thalassemia, or severe combined immunodeficiency in a country with some but still limited resources. No induction, consolidation chemotherapy is needed as would be the case for patients with acute leukemia.[15,23]

There are some limitations of this study that warrant caution in interpretation. The organizations collecting the data had neither legal enforcement to obtain nor the possibility to control all data locally for accuracy and completeness. Cross-checks with national organizations indicate that the report covers nearly 100% of all HSCTs within their country. A few countries choose not to report any data. Most missing information relates to numbers of autologous HSCTs because they are performed in some countries outside of the realm of national transplant organizations and in nonuniversity institutions. Despite these limitations, the main observations of this study regarding the main indications, donor type, transplant rates, and associations with macroeconomic factors should remain valid. Finally, we had neither information on outcome of the transplant procedures nor on correctness of the indication; this is beyond the scope of this study and would require a much longer follow-up time.[24]

This study was in part triggered by the increasing awareness by scientific and health care organizations, including the World Health Organization, to address key aspects of cell, tissue, and organ transplantation on a global level. In contrast to solid organ transplantation, HSCT faces limitations other than donor organ shortage.[25] Patients are in need of a closely matched donor, family or unrelated donor, but there are many unrelated donor registries and public cord blood banks throughout the world. In 2008, there were, for the first time, more unrelated donor HSCTs than family donor HSCTs reported to the European survey and more unrelated HSCTs across than within borders. In addition to traditional HSCT, novel treatment forms with hematopoietic stem cells for nonhematopoietic use or transplantation of nonhematopoietic stem cells for organ and tissue repair are under investigation.[26-29] The challenges with these new forms of therapy have recently been addressed; stem cell tourism has become a topic of concern.[30] Information on the current status of HSCT use has become a necessity for correct patient counseling and health care planning.

In conclusion, this global overview on HSCT activity demonstrates that it is an accepted therapy worldwide, with different needs and priorities in different regions. Transplant activity is concentrated in countries with higher health care expenditures, higher gross national income per capita, and higher team density; hence, the availability of resources, governmental support, and access to a transplant center determine regional HSCT activity.

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