High Levels of Interleukin 10 Production In Vivo Are Associated with Tolerance in SCID Patients Transplanted with HLA Mismatched Hematopoietic Stem Cells

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Summary

Transplantation of HLA mismatched hematopoietic stem cells in patients with severe combined immunodeficiency (SCID) can result in a selective engraftment of T cells of donor origin with complete immunologic reconstitution and in vivo tolerance. The latter may occur in the absence of clonal deletion of donor T lymphocytes able to recognize the host HLA antigens. The activity of these host-reactive T cells is suppressed in vivo, since no graft-vs.-host disease is observed in these human chimeras. Here it is shown that the $CD4^+$ host-reactive T cell clones isolated from a SCID patient transplanted with fetal liver stem cells produce unusually high quantities of interleukin 10 (IL-10) and very low amounts of IL-2 after antigen-specific stimulation in vitro. The specific proliferative responses of the host-reactive T cell clones were considerably enhanced in the presence of neutralizing concentrations of an anti-IL-10 monoclonal antibody, suggesting that high levels of endogenous IL-10 suppress the activity of these cells. These in vitro data correlate with observations made in vivo. Semi-quantitative polymerase chain reaction analysis carried out on freshly isolated peripheral blood mononuclear cells (PBMC) of the patient indicated that the levels of IL-10 messenger RNA (mRNA) expression were strongly enhanced, whereas IL-2 mRNA expression was much lower than that in PBMC of healthy donors. In vivo IL-10 mRNA expression was not only high in the T cells, but also in the non-T cell fraction, indicating that host cells also contributed to the high levels of IL-10 in vivo. Patient-derived monocytes were found to be major IL-10 producers. Although no circulating IL-10 could be detected, freshly isolated monocytes of the patient showed a reduced expression of class II HLA antigens. However, their capacity to stimulate T cells of normal donors in primary mixed lymphocyte cultures was within the normal range. Interestingly, similar high in vivo IL-10 mRNA expressions in the T and non-T cell compartment were also observed in three SCID patients transplanted with fetal liver stem cells and in four SCID patients transplanted with T cell–depleted haploidentical bone marrow stem cells. Taken together, these data indicate that high endogenous IL-10 production is a general phenomenon in SCID patients in whom allogeneic stem cell transplantation results in immunologic reconstitution and induction of tolerance. Both donor T cells and host accessory cells contribute to these high levels of IL-10, which would suppress the activity of host-reactive T cell in vivo.

GVHD remains the major clinical problem after allogeneic hematopoietic stem cell transplantation (1–3). Removal of donor T lymphocytes, which are the principal effectors in antihost reactivity, by T cell depletion of the graft or T cell inactivation by administration of immunosuppressive drugs can only limit, but not avoid, GVHD. Therefore, allogeneic stem cell transplantations generally result in different grades of GVHD. On the other hand, there are situations in which

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a full in vivo tolerance is obtained, despite HLA mismatches between hosts and donors. Examples are SCID patients who have been cured after HLA mismatched bone marrow (BMT)[1] or fetal liver (FLT) stem cells transplantations (4–8). In these children, a split chimerism, with T/NK cells of donor origin and B cells/monocytes of host origin is often observed, which, however, does not prevent the establishment of normal immune responses in vivo and in vitro (6–10). Furthermore, complete tolerance is achieved between donor- and host-derived cells. However, in some of these patients, donor-derived $CD8^+$ and $CD4^+$ T cells specific for class I and II HLA Ags of the host have been detected at high frequencies in vitro (11, 12). This indicates that tolerance is not due to clonal deletion of host-reactive T cells, but suggests that peripheral mechanisms are responsible for the unresponsiveness to the host cells in vivo.

Studies in mouse chimeras suggested that self- or alloantigen–specific T cells may escape clonal deletion and become anergic (13). Although it is not clear how anergic cells are regulated, it has been hypothesized that lymphokines may induce and/or maintain this nondeletional type of tolerance (14). It has been shown in animal models, that anergic cells have an altered regulation of the IL-2 pathway in response to antigens, which may account for their functional inactivation (15).

Recently, IL-10 has been identified as a critical cytokine suppressing multiple activities of the immune response (16, 17). Human IL-10 is produced by different cell types, including T cells, monocytes, and B cells, after activation (18, 19). In vitro studies have shown that IL-10 prevents Ag-specific T cell proliferation indirectly by reducing the Ag-presenting capacity of monocytes that is associated with downregulation of class II HLA expression (20). In addition, IL-10 directly suppresses T cell proliferation by specific inhibition of IL-2 production by T cells (21). Similarly, IL-10 has potent inhibitory effects on alloantigen-induced proliferative and cytotoxic T cell responses (22). IL-10 also inhibits cytokine synthesis by activated monocytes, and it downregulates its own production, suggesting that it can affect immune responses via an autoregulatory feedback mechanism (18). Overall, these biological activities indicate that IL-10 is a potent negative regulator of immunoproliferative and inflammatory responses, including alloreactivity.

In this study, we describe that the $CD4^+$ host-reactive T cell clones isolated from a SCID human chimera produce very low levels of IL-2 and unusually high amounts of IL-10, which partially inhibit their proliferation in vitro. Moreover, the amounts of IL-10 messenger RNA (mRNA) in the PBMC of the patient, as well as in the PBMC of seven other patients tested, were considerably higher than those in PBMC of normal donors. Interestingly, the highest levels of IL-10 mRNA were expressed in the non-T cell subset. Taken together, these data indicate that endogenous IL-10, produced by donor and particularly host cells, may play a key role in maintaining in vivo tolerance after allogeneic stem cell transplantation by suppressing the antihost reactivity of donor-derived T cells.

Materials and Methods

Patients. The clinical history and follow-up of patient RV have been described in detail previously (12). Briefly, the patient was diagnosed to suffer from SCID, with no T cells but normal numbers of monocytes and B cells. He received 7 FLT 6 yr ago obtained from multiple HLA mismatched donors. These transplantations resulted in a stable engraftment of T and NK cells of one donor, whereas B cells and monocytes remained of host origin. The percentages of $CD2^+$ and $CD3^+$ cells have always been in the normal range, but the CD4/CD8 ratio was constantly inverted (0.6). In addition, a high proportion (30%) of the $CD3^+$ T cells were TCR-γ/δ^+. The percentage of NK cells remained low (<4%). The patient is healthy, but there is no complete restoration of Ig production which requires Ig treatment every 3 wk.

Patients SP, MJ, and TD were also treated with FLT. Patients SP and MJ were affected with SCID and they were transplanted 17 and 11 yr ago, respectively. Their clinical history and immunological findings have already been described (8, 23). Patient TD suffered from bare lymphocyte syndrome, a genetically transmitted form of SCID, due to a lack of expression of HLA antigens. His clinical history has been previously reported (24). He received a transplant 5 yr ago in utero at 28 wk of gestational age. The FLT patients did not receive any immunosuppressive treatment before or after transplantation.

NJL, DE, TJ, and VR are SCID patients transplanted with parental haploidentical BMT, previously depleted of T cells by agglutination with soybean agglutinin and E-rosette formation. Before the transplant, they were conditioned with antithymocyte globulin, and cyclophosphamide. NJL and VR received transplants 4 and 5 yr ago, respectively, and they did not receive any immunosuppressive therapy in the past 3 yr. DE and TJ were transplanted 5 and 8 mo ago, respectively, and they are still under treatment with cyclosporin and steroids.

Establishment of T Cell Clones. $CD4^+$ host-reactive T cell clones were obtained from the PBMC of patient RV after two subsequent activations with the host's EBV-transformed lymphoblastoid cell line (EBV-LCL), in the presence of IL-2, as described (12). $CD4^+$ alloreactive T cell clones were obtained from the PBMC of the patient and from a normal donor after the same procedure, using HLA mismatched EBV-LCL as stimulator cells. The cloning was performed by limiting dilution at 0.3 cells/well in the presence of a feeder cell mixture, PHA and IL-2 (12). T cell clones were screened for Ag specificity by testing proliferation and IFN-γ production in response to the host-derived or allogeneic EBV-LCL, as previously described (12). T cell clones were maintained in culture by weekly restimulation with the feeder cell mixture and PHA. Between stimulations with feeder cells, the T cell clones were expanded with IL-2.

Reagents. Purified recombinant human IL-10 was provided by Schering-Plough Research (Bloomfield, NJ). The blocking anti-IL-10 mAb 19F1 has been described (25). The anti-IL-2 receptor mAb B-B10 and anti-CD3 mAb SPV-T3 were previously described (26, 27). FITC or PE-conjugated, anti-CD3 (anti-Leu4), anti-CD14 (anti-LeuM3), anti-CD16 (anti-Leu11a), anti-CD19 (anti-Leu12), anti-CD20 (anti-Leu16), anti-CD56 (anti-Leu19), anti-HLA-DR (anti-HLA-DR) mAbs and control mAbs of the appropriate iso-

[1] *Abbreviations used in this paper:* FLT, fetal liver stem cell transplantation; BMT, bone marrow stem cell transplantation; EBV-LCL, EBV-transformed-lymphoblastoid cell line; HPRT, hypoxanthine phosphoribosyltransferase; MFI, mean fluorescence intensity; mRNA, messenger RNA.

types were purchased from Becton Dickinson & Co. (Mountain View, CA). Magnetic beads (Dynabeads M-450; Dynal, Oslo, Norway) were used at a bead to cell ratio of 5:1 to obtain purified normal donor T cells negatively depleted of $CD14^+$, $CD16^+$, $CD19^+$, and $CD56^+$ cells, as described by the supplier.

Proliferation Assays. T cell clones (2×10^4 cells/well) or purified T cells (10^5 cells/well) were stimulated with purified monocytes (4×10^4 cells/well) in 200-μl in flat-bottomed 96-well plates. The monocytes of patient RV and of normal donors 1 and 2 were isolated from PBMC by sorting $CD14^+$ cells (purity >99%). For another series of experiments, monocytes of normal donors were isolated from PBMC by density centrifugation in a blood component separator, followed by centrifugal elutriation (18). These monocyte preparations, which were 95% pure, had either the HLA-DR phenotype of the host, or that of the allogeneic EBV-LCL that had been used to generate the T cell clones. For measurement of the proliferation of T cell clones, cells were cultured for 72 h at 37°C in 5% CO_2, and subsequently pulsed with [^{3}H]TdR for 6 h, and harvested as described previously (12). Proliferation of purified T cells was measured after incubation period of 4 d, followed by [^{3}H]TdR incorporation for 12 h. All tests were carried out in triplicate.

Primers and Probes. The following primers were used for PCR analysis: hypoxanthine phosphoribosyltransferase (HPRT) sense primer 5'-TATGGACAGGACTGAACGTCTTGC-3'; HPRT antisense primer 5'-GACACAAACATGATTCAAATCCCTGA-3'; IL-2 sense 5'-AAGAATCCCAAACTCACCAGGATGC-3'; IL-2 antisense 5'-CCCTTTAGTTCCAGAACTATTACGT-3'; IL-10 sense 5'-ATGCCCCAAGCTGAGAACCAAGACCCA-3'; IL-10 antisense 5'-TCTCAAGGGGCTGGGTCAGCTATCCCA-3' (28); IFN-γ sense 5'-ATGAAATATACAAGTTATATCTTGGCTTT-3'; and IFN-γ antisense 5'-GATGCTCTTCGACCTCGAAACAGCAT-3'. The following oligonucleotides were used for Southern analysis of PCR products: HPRT 5'-GTCCCCTGTTGACTGGTCATTACAAT-3'; IL-2 5'-CATGCCCAAGAAGGCCACAGAACTGAAACA-3'; IL-10 5'-CAGGTGAAGAATGCCTTTAATAAGCTCCAAGAGAAAGGCATCTACAAAGCCATGAGTGAGTTTGACATC-3' (28); and IFN-γ 5'-TGACTAATTATTCGGTAACTGACTTGAATG-3'.

RNA Isolation and PCR Analysis. Total RNA was isolated from PBMC or sorted cell populations by RNA isolation solvent (RNAzol B; TEL-TEST, Inc., Friendswood, Texas). The whole procedure was performed as described for mouse cytokines (29, 30). cDNA was prepared in duplicate by reverse transcription using SuperScript (GIBCO-BRL, Gaithersburg, MD) according to the specifications of the supplier. Each cDNA was amplified by PCR in the presence of HPRT, a housekeeping enzyme, or lymphokine-specific primers. As reference, 11 twofold dilutions of a standard cDNA were included. The standard sample was cDNA obtained from ConA-activated $CD4^+$ T cell clone B21. PCR amplification for cytokine-specific primers was performed in 50 μl with 1 U of Taq polymerase (Perkin-Elmer Corp., Emeryville, CA) by a 96-well thermocycler (GeneAmp PCR System 9600; Perkin-Elmer Corp.) under the following conditions: 30 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C. Subsequently, 10 μl of the amplified product was dot-blotted in duplicate to Nytran membrane (Schleicher & Schuell, Inc., Keene, NH) using a 96-well manifold (Bio-Rad Laboratories, Richmond, CA). Oligonucleotide probes specific for a sequence internal to the primers used in the amplification, were ^{32}P end-labeled and hybridized to the membranes. For quantification of the signal, the membranes were scanned by a Phosforimager (Molecular Dynamics, Sunnyvale, CA). All sample values were within the range of the reference curve. HPRT and cytokine mRNA values were determined based on the reference titration curve. Calculations were performed using ELISA software (Softmax; Molecular Devices, Inc., Menlo Park, CA). Values of each sample were expressed as ratio between the lymphokine value and the HPRT value using the following formula: 100× [(mean of cpm of PCR for cytokine/mean of cpm of PCR for HPRT)].

Immunofluorescence Analysis. For detection of cell surface Ags, 10^5 cells were labeled with FITC or PE-conjugated mAbs. Cells were incubated for 30 min with the appropriate mAb at 4°C in PBS with 0.1% BSA and 0.02 mM NaN_3. After three washes, the label cell samples were analyzed on a FACScan® (Becton Dickinson & Co.). Where indicated, fluorescence of the cells is expressed as channel number, on a logarithmic scale, representing mean fluorescence intensity (MFI) of positive cells. The MFI reflects the Ag density per cell.

For cell sorting, PBMC were labeled according to the same procedure in PBS with 5% FCS and sorted by FACStar® or Vantage (Becton Dickinson & Co.). The purity of sorted T cell, non-T cell, monocytes, and B cell fractions was >99.2%. The sorted cell populations were immediately used for mRNA extraction.

Determination of Lymphokine Production. Resting T cell clones were collected 8–10 d after activation with the feeder cell mixture, washed, and stimulated with the host-derived or allogeneic EBV-LCL (1:1 ratio) or in the presence of Con A (10 μg/ml) or TPA (1 ng/ml) plus anti-CD3 mAb (0.1 μg/ml) or TPA (1 ng/ml) plus Ca^{2+} ionophore (A23187, 500 ng/ml). Supernatants were harvested after incubation for 24 h. For kinetics of lymphokine production, supernatants were collected 1, 4, 8, 12, 36, 48, and 72 h after stimulation. The presence of IL-2 and IL-10 was quantified by immunoenzymetric assays performed as previously described (25). Sensitivity of these assays was 20 pg/ml for IL-2, and 50 pg/ml for IL-10.

Results

IL-2 and IL-10 Production by Host-reactive T Cell Clones. We previously showed that donor-derived $CD4^+$ T cell clones, obtained from the PBMC of human chimeras, proliferate and produce normal amounts of IFN-γ in response to host cells that are HLA incompatible (12). Here, we investigated the ability of host-reactive T cell clones isolated from patient RV, to produce IL-2 and IL-10 after activation by the EBV-LCL of host origin. In Fig. 1 *A* it is shown that 29 of the patient's $CD4^+$ T cell clones produced very low levels of IL-2 (mean ± SE = 156 ± 40 pg/ml) after Ag-specific stimulation. In contrast, IL-10 was produced at unusual high levels (15,719 ± 2,226 pg/ml). Both cytokines were undetectable in supernatants of unstimulated T cell clones. IL-10 production by the EBV-LCL used as stimulator cells was in all cases <500 pg/ml, whereas these cells, as expected, did not produce IL-2 (data not shown). A similar pattern of high IL-10 (14,495 ± 906 pg/ml) and very low IL-2 production (179 ± 61 pg/ml) was also observed, when the T cell clones were activated by TPA plus anti-CD3 mAb (Fig. 1 *C*) which has been shown to induce optimal IL-10 synthesis (19). In contrast, activation by Con A (Fig. 1 *B*) or TPA plus Ca^{2+} ionophore (Fig. 1 *D*) resulted in normal levels of IL-2 production by the host-reactive T cell clones (756 ± 194 and 6,697 ± 975 pg/ml respectively). However, IL-10 production was decreased after stimulation with Con A (2,462 ± 593 pg/ml) and to a lesser extent after TPA plus Ca^{2+} ionophore activation (11,624 ±

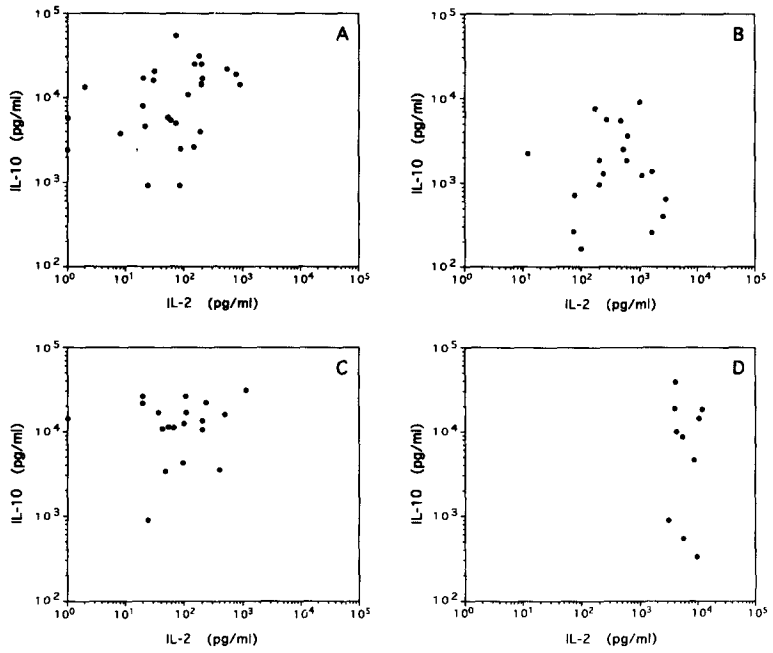

Figure 1. IL-10 and IL-2 production by host-reactive CD4+ T cell clones (●) of patient RV, after Ag-specific activation with the host EBV-LCL (*A*), polyclonal activation with Con A (*B*), TPA plus anti-CD3 mAb (*C*), and TPA plus Ca^{2+} ionophore (*D*). See Results for the mean values.

3,779 pg/ml), both of which have been shown to be poor IL-10 inducers (19).

The low levels of IL-2 in supernatants of Ag-activated host-reactive T cell clones were not due to IL-2 consumption by the proliferating T cell clones, since addition of anti-IL-2 receptor mAb did not result in increased IL-2 levels (data not shown).

High IL-10 Production Is Specific for Host-reactive T Cell Clones. To determine whether high IL-10 production in response to HLA Ags is a specific property of host-reactive T cell clones, we compared the amounts of IL-10 produced by the host-reactive T cell clones of patient RV with those of normal alloreactive CD4+ T cell clones isolated from PBMC of the patient and from PBMC of a normal donor. Four different allogeneic HLA mismatched EBV-LCL were used as stimulator cells. As shown in Table 1, both patient- and normal donor-derived, alloreactive T cell clones produced significantly lower levels of IL-10 compared to those produced by host-reactive T cell clones. This indicates that high IL-10 production is a specific property of host-reactive T cell clones. The alloreactive CD4+ T cell clones of the normal donor

Table 1. *IL-10 Production after Antigen-specific Stimulation* of T Cell Clones Obtained from a Human Chimera (RV) and from a Normal Donor (ND)*

T cell clones	No. tested	IL-10 (pg/ml) Mean values	SE
Host-reactive (RV)	29	15,719	2,226
Alloreactive (RV)	23	6,067‡	881
Alloreactive (ND)	23	6,900§	1,734

* EBV-LCL were used as stimulators.
‡ $p = 0.005$ determined by Student's t test analysis.
§ $p = 0.02$ determined by Student's t test analysis.

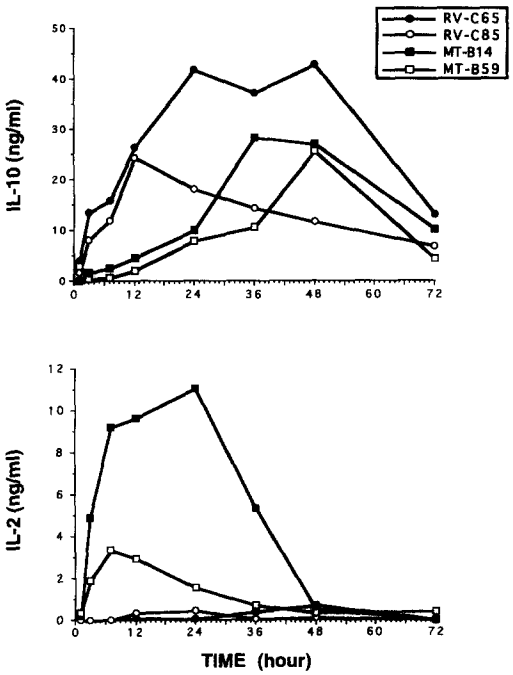

Figure 2. Kinetics of IL-10 and IL-2 production by two host-reactive CD4+ T cell clones (RV-C65 and RV-C85) isolated from patient RV and two alloreactive CD4+ T cell clones (MT-B14 and MT-B59) isolated from a normal donor. The alloreactive T cell clones selected as control were considered high IL-10 producers. For both sets of T cell clones, the supernatants were harvested at different times (1, 4, 8, 12, 24, 36, 48, and 72 h) after Ag-specific activation.

produced normal amounts of IL-2 (5,428 ± 919 pg/ml), whereas the alloreactive CD4+ T cell clones from the patient synthesized variable amounts of IL-2 (398 ± 94 pg/ml), which overall were not significantly higher than those produced by host-reactive T cell clones. This suggests that low IL-2 production may be a general property of donor-derived T cells, irrespective of whether they are host-reactive or specific for third-party HLA Ags.

Kinetics of IL-2 and IL-10 Production by Host-reactive T Cell Clones. The kinetics of IL-2 and IL-10 production by CD4+ T cell clones are different. IL-2 is detectable very early in the supernatant of activated T cell clones, with a peak of production generally occurring after 8–12 h, whereas IL-10 is synthesized late after stimulation: it starts to appear 8 h after activation and reaches the highest levels 24–48 h later (17). The kinetics of IL-2 and IL-10 production by host-reactive CD4+ T cell clones and alloreactive T cell clones, obtained from a normal donor, were investigated after stimulation by specific HLA antigens presented by EBV-LCL (Fig. 2). To study the kinetics of IL-10 optimally, high IL-10–producing control alloreactive T cell clones were selected. The alloreactive T cell clones of the normal donor (MT-B14 and MT-B59) produced IL-10 at low levels within the first 12 h of culture. IL-10 increased progressively and reached peak values at 36–48 h after activation. Similar kinetics have been reported for Ag-specific CD4+ T cell clones (17). Interestingly, significant levels of IL-10 were already present in the supernatant of host-reactive T cell clones (RV-C65 and RV-C85) at 4 h after activation. Thereafter, IL-10 levels increased rapidly and the highest concentrations were obtained 12–24 h after activation. IL-2 production by the control CD4+ T cell clones showed normal kinetics, since considerable levels of IL-2 could be detected 3 h after activation. In contrast, low levels of IL-2 started to be detected in the supernatants of host-reactive T cell clones only 12 h after activation. In T cell clone RV-C85, the highest concentrations of IL-2 were detectable between 12 and 24 h, and in T cell clone RV-C65, between 36 and 48 h after activation. No differences were observed between the two sets of clones in the kinetics of production of other lymphokines, such as IFN-γ (data not shown). These results illustrate the specific kinetics of Ag-induced IL-2 and IL-10 synthesis by host-reactive T cell clones in which IL-2 production is preceded by that of IL-10.

Effect of Endogenous and Exogenous IL-10 on Ag-specific Proliferation. IL-10 inhibits (allo)antigen-specific T cell proliferation in the presence of monocytes as APC (20, 22). To investigate whether endogenous IL-10 production could contribute to the reduced proliferation of host-reactive T cell clones, their proliferative responses were analyzed in the presence of different concentrations of anti-IL-10 mAb. Alloreactive control T cell clones obtained from a healthy donor, were tested in parallel. For these experiments, monocytes expressing the relevant HLA Ag of the host were used as stimulators. In Table 2, it is shown that the antigen-specific proliferative responses of host-, but not of alloreactive T cell clones, were significantly ($p \leq 0.005$) increased in the presence of anti-IL-10 mAb (mean percentages of increase, 39, 50, and 57 for the three different concentrations of mAb). This indicates that IL-10 produced by the host-reactive T cell clones suppresses proliferation of these cells in vitro. In addition, proliferation of the host-reactive T cell clones was further inhibited in the presence of exogenous IL-10 (Table 2). IL-10 inhibited the proliferative responses of the control CD4+ alloreactive T cell clones to a similar extent (Table 2).

Table 2. *Antigen-specific Proliferative Responses of T Cell Clones in the Presence of Anti-IL-10 mAb or of Exogenous IL-10**

T cell clones	Medium	Anti-IL-10 0.05 μg/ml	Anti-IL-10 0.5 μg/ml	Anti-IL-10 5 μg/ml	IL-10 1 U/ml	IL-10 10 U/ml	IL-10 100 U/ml
Host-reactive							
RV-B 11	11.9 ± 1.1‡	18.9 ± 0.9	19.1 ± 0.8	21.2 ± 1.9	6.2 ± 0.8	1.4 ± 0.2	1.3 ± 0.2
RV-C 15	4.8 ± 0.2	6.0 ± 0.3	5.8 ± 0.2	6.1 ± 0.3	2.9 ± 0.4	1.0 ± 0.1	0.6 ± 0.0
RV-C 30	15.5 ± 0.1	19.9 ± 0.1	22.0 ± 1.1	20.7 ± 0.9	15.7 ± 0.7	8.3 ± 0.2	4.2 ± 0.5
RV-C 65	5.9 ± 0.1	10.1 ± 0.1	11.4 ± 0.4	11.8 ± 0.7	5.0 ± 0.7	1.9 ± 0.1	1.2 ± 0.2
RV-C 85	5.1 ± 0.3	5.8 ± 0.7	6.7 ± 0.1	7.4 ± 0.5	3.3 ± 0.3	0.9 ± 0.1	0.4 ± 0.0
Alloreactive							
MT-B 13	149.9 ± 7.0	120.4 ± 6.3	131.2 ± 24.4	138.2 ± 15.2	117.1 ± 25.0	53.9 ± 3.0	52.2 ± 4.0
MT-B 46	72.6 ± 2.8	70.2 ± 2.3	68.6 ± 0.2	68.6 ± 0.4	51.3 ± 1.6	33.3 ± 1.4	30.8 ± 1.1
MT-B 49	98.2 ± 2.9	91.9 ± 9.2	86.7 ± 9.6	80.0 ± 2.9	68.9 ± 7.0	35.2 ± 1.0	30.8 ± 1.4
MT-B 51	171.4 ± 13.4	167.2 ± 19.4	168.7 ± 17.4	179.9 ± 23.7	163.1 ± 2.0	109.6 ± 9.1	103.8 ± 10.6
MT-B 59	83.1 ± 6.1	86.4 ± 2.4	89.9 ± 3.8	85.3 ± 2.3	54.8 ± 7.8	21.4 ± 1.9	18.0 ± 1.9

* Monocytes expressing the relevant HLA antigens were used as stimulators.
‡ [^{3}H]TdR incorporation cpm × 10^{-3} ± SD.

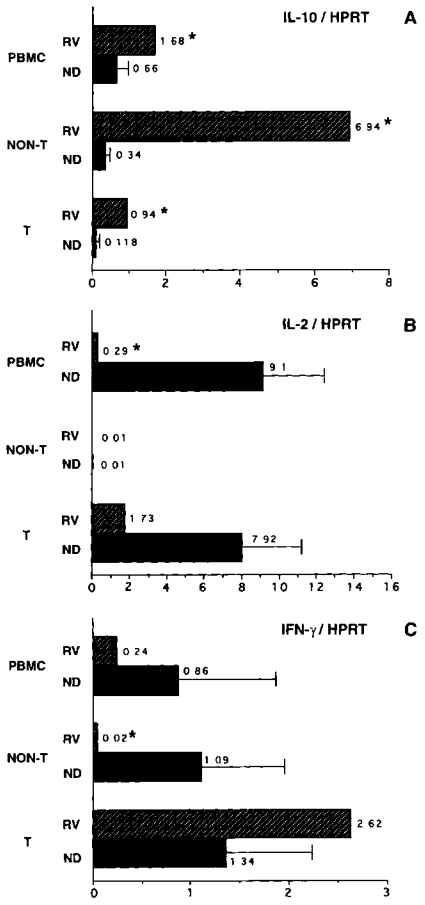

Figure 3. PCR determination of IL-10 (*A*), IL-2 (*B*), and IFN-γ (*C*) mRNA expression in patient's (RV) and normal donors' (ND) total PBMC and sorted T and non-T cell populations. Values are expressed as the ratio between the lymphokine and HPRT (see Materials and Methods). Values referred to the patient are representative of results obtained from three different experiments performed on three PBMC samples; data referred to ND represent the mean values obtained from five subjects (± SD). (*) A value that differs from the mean value of ND ± 2 SD and that is considered statistically significant.

Expression of IL-10 mRNA In Vivo. Since host-reactive T cells are present at high frequencies in the PBMC of patient RV (12), we hypothesized that IL-10, which is strongly produced by Ag activated host-reactive T cells in vitro, should be detectable in vivo, where donor-derived, host-reactive T cells continuously and specifically interact with the host cells. Therefore, IL-10 mRNA expression in the patient's PBMC was determined by using a semiquantitative PCR analysis. IL-10, IL-2, and IFN-γ mRNA levels of the patient's PBMC were compared with those of normal donors in the absence of activation. The results shown in Fig. 3 *A* indicate that IL-10 mRNA expression in the PBMC of patient RV was significantly higher than the mean levels of IL-10 mRNA present in the PBMC of five normal donors tested in parallel. PCR analysis of sorted $CD3^+$ T cells and non-T cells ($CD14^+CD16^+CD20^+$) showed that IL-10 mRNA expression in both fractions was significantly higher in the patient than in the normal donors. A remarkable high IL-10 mRNA expression was detected in the non-T cell subset. PCR data obtained from purified $CD14^+$ monocytes and $CD20^+$ B cells showed that IL-10 mRNA is mainly expressed by monocytes, whereas B cells were negative, or expressed very low levels of IL-10 mRNA. In addition, IL-10 mRNA expression in the patient's monocytes was much higher (IL-10/HPRT ratio, 8.5) than in that of the normal donors (IL-10/HPRT ratio, 0.8 ± 0.3). IL-2 mRNA (Fig. 3 *B*) in PBMC and in T cells of patient RV was decreased compared to that of normal donors, although this decrease was more significant in total PBMC than in the sorted T cell subset. IL-2 mRNA was not detectable in non-T cell subsets. IFN-γ mRNA expression (Fig. 3 *C*) in the PBMC and in T cells of the patient and of normal donors were comparable. In contrast, the expression of IFN-γ mRNA was low in the non-T cells of the patient. This is in line with the observation that the amount of IFN-γ present in the non-T cell subset seems to be related to the proportion of $CD16^+$ cells (Bacchetta, R., et al., unpublished data), which was very low (<4%) in the patient as compared to the normal donors (4–10%). Thus in patient RV, IL-10 is highly expressed in vivo by both donor-derived T cells and host-derived monocytes. This is consistent with the observation that spontaneous IL-10 production in vitro by the patient's PBMC was also high, median, 3,214 pg/ml, vs. the 518 pg/ml previously observed in a series of normal donors (25), despite the fact that the percentages of $CD3^+$ T cells, $CD20^+$ B cells, and $CD14^+$ monocytes in the patient's PBMC were comparable to those of healthy donors.

To investigate whether high levels of IL-10 production in vivo are a general phenomenon in SCID patients after allogeneic stem cell transplantation, we measured the IL-10 mRNA expression in seven other SCID patients with split chimerism. Three were reconstituted with fully HLA mismatched FLT and four with haploidentical BMT. In all patients, immunotolerance was achieved with stable engraftment of T cells derived from the donor, and B cells/monocytes of host origin. Results shown in Fig. 4 indicate that IL-10 mRNA expression was significantly higher in the PBMC of patients compared to 13 normal donors tested ($p = 0.006$). The higher IL-10 expression was present in both the donor-derived T cells ($p < 0.001$) and the host-derived non-T cells ($p = 0.001$). No remarkable differences were observed between patients transplanted with FLT or BMT. These results demonstrate that high levels of IL-10 are consistently present in chimeric patients after allogeneic stem cell transplantation.

Reduced HLA-DR Expression on Monocytes In Vivo. IL-10 downregulates constitutive and IL-4- or IFN-γ–induced class II HLA expression on APC in vitro (20). To determine whether high endogenous IL-10 production would also result in a reduced expression of class II HLA antigens in vivo, monocytes of patient RV were analyzed. Indeed, the MFI of HLA-DR expression on $CD14^+$ monocytes freshly isolated from the patient was lower than that of monocytes of normal donors (Fig. 5). In addition, the reduced HLA-DR expression on the patient's monocytes did not change when these cells were incubated in medium for 48 h. In contrast, HLA-DR expression on control monocytes from healthy donors further increased twofold after this culture period.

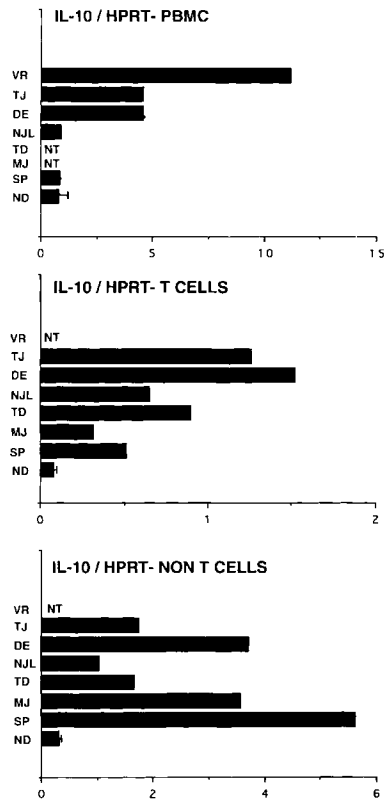

Figure 4. PCR determination of IL-10 mRNA expression in total PBMC and sorted T and non-T cell populations of SCID patients transplanted with haploidentical BMT (*VR, TJ, DE, NJL*) or fully HLA mismatched FLT (*TD, MJ, SP*), and of 13 normal donors (*ND*). Values are expressed as the ratio between IL-10 and HPRT (see Materials and Methods). (*NT*) Not tested. Statistical analysis was performed by unpaired Student's *t* test; *p* values between patients and normal donors were the following: PBMC, $p = 0.006$; T cells, $p < 0.001$; non-T cells, $p = 0.001$.

HLA-DR expression doubled when both the patient's and control monocytes were incubated in the presence of anti-IL-10 mAb, but HLA-DR expression on the patient's monocytes never reached the level of that of the normal donors'

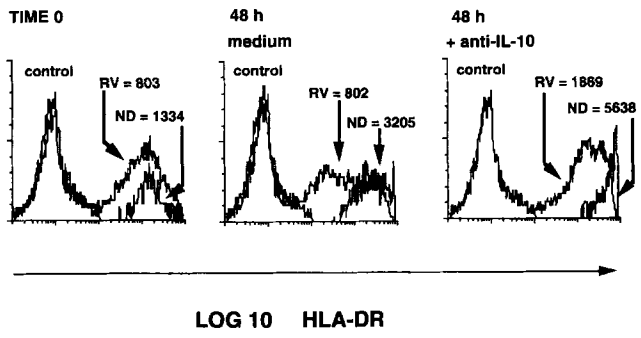

Figure 5. MFI of HLA-DR expression on CD14$^+$ monocytes of patient RV and of normal donors (*ND*). One representative experiment out of three performed is shown. The phenotype was done on freshly isolated PBMC (*TIME 0*) and after 48 h of incubation in medium or in medium + anti-IL-10 mAb (5 μg/ml). (*Control*) Cells stained with isotypic control mAbs.

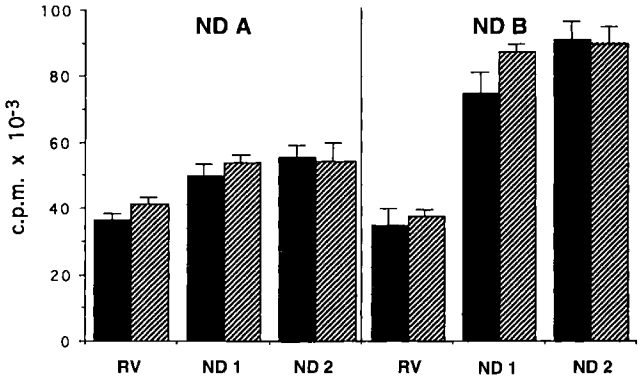

Figure 6. Proliferative responses of T cells isolated from two normal donors (*ND A* and *ND B*) toward sorted CD14$^+$ monocytes of patient RV and of two normal donors (*ND1* and *ND2*), in the absence (■) or in the presence (■) of anti-IL-10 mAb (20 μg/ml). Values are expressed as cpm × 10^{-3} and represent the mean [^{3}H]TdR incorporation of triplicate cultures ± SD. Background cpm of cells cultured in medium alone were subtracted.

monocytes. No difference in the HLA-DR expression on B cells was observed between patient RV and normal donors either freshly isolated or after 48 h of culture (data not shown).

Stimulatory Capacity of the Patient's Monocytes. The IL-10–induced downregulation of class II HLA expression on monocytes has been associated with their reduced capacity to present Ag and to induce Ag-specific T cell proliferation in vitro (20). Therefore, the Ag-presenting capacity of the monocytes of patient RV was investigated. In Fig. 6 it is shown that, although the proliferative responses of T cells from normal donors toward the patient's sorted CD14$^+$ monocytes are somewhat lower than those toward the two controls' sorted CD14$^+$ monocytes, they are within the normal range. In addition, the proliferative responses were not significantly increased in the presence of neutralizing concentrations of anti-IL-10 mAb. These results indicate that under the present culture conditions, the overall stimulatory capacity of the patient's monocytes was not impaired when normal T cells were used as responders. In addition, no inhibitory effect of endogenous IL-10 was observed, suggesting that this effect could be overcome by the relatively high levels of IL-2 produced by normal T cells during allogeneic responses.

Discussion

Transplantation of HLA mismatched hematopoietic stem cells in patients suffering from SCID may result in a selective T lymphocyte chimerism (5, 8). In these split human chimeras, immunologic reconstitution can be achieved with tolerance between donor and host cells. The lack of in vivo GVHD and the in vitro unresponsiveness to the host cells in primary MLCs, indicate that donor-derived T lymphocytes develop tolerance to HLA antigens uniquely expressed on recipient cells (12, 31, 32). This tolerance is not entirely due to clonal deletion, since host-reactive T cells can be isolated at high frequencies from the peripheral blood of SCID patients reconstituted either with fully mismatched fetal liver stem cells

(11, 12) or with haploidentical bone marrow (Bacchetta, R., et al., manuscript in preparation). Therefore, a peripheral autoregulatory mechanism should be responsible for the in vivo homeostasis.

In this study, we demonstrate that donor-derived $CD4^+$ host-reactive T cell clones, isolated from one of such a human chimeras, produced unusually high amounts of IL-10 after Ag-specific activation. This property is unique for host-reactive T cell clones, since control alloreactive T cell clones, isolated from both the patient and normal donors, produced normal levels of IL-10 (19). Host-reactive T cell clones also have distinct kinetics of IL-10 secretion which can be detected in the supernatants at high concentrations very early after Ag activation, whereas normally IL-10 is produced late after stimulation (17). In addition, the Ag-specific proliferative responses of the host-reactive T cell clones, unlike those of alloreactive T cell clones isolated from normal donors, were increased in the presence of anti-IL-10 mAb, indicating that the endogenous IL-10 production plays a role in suppressing the proliferation of these cells. In contrast, $CD4^+$ host-reactive T cell clones produced very low amounts of IL-2, especially after specific activation with Ag or with TPA plus anti-CD3 mAb. However, the host-reactive T cell clones produced normal levels of IL-2 after polyclonal activation by Con A or TPA plus Ca^{2+} ionophore, indicating that low IL-2 production is not an intrinsic property of these cells, but occurs only after Ag-specific or TCR-mediated stimulation. Indeed, the alloreactive T cell clones isolated from the same patient, also produced low levels of IL-2 after Ag-specific activation, suggesting that low IL-2 production may not be a specific property of host-reactive T cell clones. IFN-γ production by the host-reactive T cell clones was comparable to that of alloreactive T cell clones, independently of their mode of activation (12).

In line with the observations at the clonal level, we found higher levels of IL-10 mRNA expressed in the patient's PBMC than in those of normal donors. Separation of the PBMC in purified T and non-T cell populations indicated that both cell fractions expressed high levels of IL-10 mRNA, although IL-10 mRNA was considerably more enhanced in the non-T cell compartment. Purified B cells expressed no, or low levels of IL-10 mRNA, whereas high expression of IL-10 mRNA was detected in the monocytes of the patient. IL-2 mRNA expression was much lower in the patient than in normal donors, which is compatible with the results obtained with both host- and allo-reactive T cell clones, and suggests that low IL-2 production may be a general characteristic of the patient's T cell compartment. IFN-γ mRNA expression in the patient was comparable to that of normal donors, with the exception that non-T cells of patient had low levels of IFN-γ mRNA, which was probably due to the very low proportion of NK cells present in the patient (12).

Interestingly, high endogenous IL-10 production was not restricted to patient RV, but was observed in seven other chimeras, three of whom had been transplanted with fetal liver cells and four with haploidentical bone marrow cells. Also in these patients, high levels of IL-10 mRNA expression were present in the donor-derived T and in the non-T cell fractions which remained of host origin after transplantation.

Collectively, these data show a clear distinctive pattern in both kinetics and levels of in vivo and in vitro IL-10 and IL-2 production in a human chimera and demonstrates that the high endogenous IL-10 production suppresses the activity of the host-reactive T cells in vitro. In addition, they indicate that high endogenous IL-10 production is a general phenomenon occurring in SCID patients successfully transplanted with HLA mismatched stem cells and in whom tolerance is achieved.

IL-10 has multiple suppressive effects on various effector phases of the immune response (16, 17). In humans, IL-10 was found to prevent Ag-specific proliferative T-cell responses by reducing the antigen-presenting capacity of APCs that were associated with downregulation of class II MHC on these cells (20). IL-10 also inhibited the synthesis of most monokines (18). Furthermore, IL-10 has a direct inhibitory effect on T cell proliferation by specifically downregulating IL-2 mRNA expression and IL-2 production by PBMC, T cells, and T cell clones belonging to different Th subsets (21). We recently described that exogenous IL-10 also acts as a potent inhibitory factor for cytotoxic and proliferative alloresponses in a one-way primary MLC. In addition, the presence of anti-IL-10 mAb enhances the alloreactive proliferative responses, indicating that endogenous IL-10 production modulates allogeneic responses in MLCs (22).

Based on these biological activities, we hypothesize that the endogenous IL-10 production in our transplanted patients is responsible for maintaining tolerance in vivo. IL-10 may inhibit the development of GVHD by preventing optimal activation and IL-2 synthesis by host-reactive T cells. In addition, it may suppress the antigen-presenting capacity and cytokine production by host cells. Several studies in mice (33, 34) and human (35, 36) indicate that cytokines play a major role in acute GVHD, not only by facilitating T cell expansion but also by directly causing tissue damage. High serum levels of IL-2, IFN-γ, IL-1, and TNF-α have been correlated with the severity of acute GVHD, and administration of Abs which block the effects of these cytokines can prevent and/or arrest GVHD (37). On the other hand, altered IL-2 induction pathways have been reported to be responsible for the acquisition of peripheral anergy (14, 15, 38, 39).

The signals which trigger IL-10 production in transplanted SCID patients remain to be identified. However, resting T cells and monocytes do not produce IL-10, which requires activation of these cells (19). This implies that high IL-10 production in vivo reflects a chronic activation of donor T cells and host monocytes because of ongoing immune response. One could envisage that the continuous interactions between host-reactive T cells and host HLA antigens presented by monocytes in vivo result in mutual activation of these cells and the rapid production of high levels of IL-10 locally. These high local levels of IL-10 prevent the host-reactive T cells to proliferate and produce IL-2, whereas, in an autoregulatory fashion, they simultaneously inhibit the production of proinflammatory cytokines by the monocytes and reduce class II HLA expression on these cells, thereby modifying their

antigen-presenting capacity. Whether this modified antigen-presenting capacity of the monocytes results in a particularly aberrant mode of T cell activation which contributes to the high IL-10 production by the host-reactive T cells, remains to be determined.

In the proposed model, endogenous IL-10 acts mainly locally as an antigen-specific suppressor factor which inhibits activation and proliferation of donor-derived T cells able to recognize the HLA antigens of the host. This notion is supported by the observation that no significant levels of circulating IL-10 could be detected in these patients. However, we have indirect evidence that endogenous IL-10 also may have systemic effects on cells of the immune system in patient RV, as is illustrated by the low IL-2 mRNA expression in all T cells, the reduced IL-2 synthesis by alloreactive T cell clones, and the reduction of class II HLA expression on circulating monocytes. This reduced class II HLA expression could be partially restored after in vitro incubation of the monocytes in the presence of anti-IL-10 mAb, indicating that IL-10, which has been shown to downregulate constitutive class II HLA expression on monocytes in vitro, is probably also responsible for the downregulation of these antigens in vivo. Preliminary in vitro experiments have shown that monocytes pretreated with IL-10 remain refractory for induction of class II HLA antigens by IFN-γ and IL-4 for extended periods (de Waal Malefyt, R., unpublished observations). Therefore, although endogenous IL-10 production may have its strongest impact locally on host-reactive T cells, weaker systemic effects on all T cells and monocytes probably occur concomitantly. When this scenario is correct, it predicts that these patients are constantly in a state of mild "natural" immunosuppression.

Several observations are against this hypothesis. First, all patients are apparently in good health. In addition, their peripheral blood T cells were found to respond normally to allogeneic stimulations in primary MLCs (12) or to exogenous antigens such as tetanus toxoid (Roncarolo, M.-G., unpublished observation). These results suggest that, despite the fact that alloreactive T cell clones of patient RV produced low levels of IL-2, donor T cells overall produce enough IL-2 in vivo to guarantee normal immune responses. In fact it has been reported that T cell clones in general produce 10-fold less IL-2 than freshly isolated T cells after activation (18). Furthermore, the monocytes of patient RV, despite their reduced class II HLA expression, induced proliferative responses of T cells of normal donors that were somewhat lower to those obtained with control monocytes, but which were definitely within the normal range. Moreover, under these culture conditions, anti-IL-10 mAb did not significantly enhance the proliferative responses of the normal T cells, suggesting that normal levels of IL-2 production by these cells can overcome the suppressive effects of IL-10 in vitro. These findings further support the notion that sufficient levels of IL-2 produced in vivo by the donor T cells, after activation with infectious agents, such as bacteria or viruses, can counteract the suppressive effects of IL-10, which allow normal immunoprotection without breaking the state of tolerance that would be mainly controlled locally by IL-10.

Collectively, our data provide a new insight in the importance of IL-10 produced in vivo by accessory cells and T cells for induction and maintenance of tolerance after allogeneic stem cell transplantation. In addition, they indicate that IL-10 may have potential clinical utility in preventing or reducing GVHD and allograft rejection.

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