Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-energized donor T Cells

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INTRODUCTION

The long-term benefit and wide applicability of T-cell-depleted (TCD) haploidentical-HSC transplantation (haplo-HSCT), have been limited by slow immune reconstitution and high relapse rate (1–6). Infusion of unmanipulated donor lymphocyte (DLI) to reconstitute the immune system without GvHD after HSCT (8, 9). For example, chimeric antigen-receptor modified T cells to directly treat leukemia or suicide-gene expressing T cells have been successfully employed (9–12). In addition, freshly isolated or expanded CD4⁺CD25⁺ T regulatory (Treg) cells have been used as adjuvant therapy to suppress GvHD after allogeneic HSCT in patients with hematologic malignancies (13–15). These Treg cell therapies are safe, although tested in a small number of patients, but their efficacy is still controversial. In addition, many open questions remain on the best source of Treg cells to be administered, the optimal cell dose, the survival and behavior of these cells once they are in the host environment, and their mechanism of action.

Peripheral T regulatory type 1 (Tr1) cells, with alloantigen (Allo-Ag)-specific suppressor function, have been consistently associated to a state of tolerance in chimeric patients after allogeneic HSCT. In addition, several preclinical studies demonstrated...
that Tr1 cells play a major role in the induction and maintenance of transplantation tolerance (16–21). Tr1 cells are characterized by a unique cytokine production profile (IL-10+IL-4−IL-17−) (17, 21), and by the co-expression of CD49b and LAG-3 (22). Tr1 cells control immune responses by secreting high levels of IL-10, and by killing myeloid cells (17, 23). Allo-Ag-specific Tr1 cells can be induced in vitro in the presence of IL-10 (17, 24, 25). Activation of T cells by Allo-Ags in the presence of IL-10 induces Ag-specific hypo-responsiveness, identified as T-cell anergy (26, 27). These IL-10-energized T cells contain Tr1 cells, as demonstrated at single-cell level (17), but also memory T cells able to proliferate in response to nominal Ags, such as tetanus toxoid and Candida albicans, and viral Ags, such as Epstein-Barr virus (25). In a murine model of allogeneic HSCT, transfer of IL-10-energized T cells prevented GVHD (28). These findings provided the rationale for the clinical use of IL-10-energized donor T cells (IL-10-DLI) to support immune reconstitution without severe GVHD after allo-HSCT.

We developed a GMP-validated protocol to obtain IL-10-DLI, from haploidentical hematopoietic stem cell donors (25, 29). In a small proof-of-concept clinical trial (ALT-TEN trial), 12 patients who received TCD haplo-HSCT were treated with IL-10-DLI. Here, we describe the positive outcome of TCD haplo-HSCT combined with IL-10-DLI cell therapy in four patients with high-risk/advanced stage hematologic malignancies. These patients achieved full donor chimerism, immune reconstitution, and long-term persistent disease remission. Cells from all four patients expressed biomarkers consistent with tolerance and Tr1 cell enrichment. This study indicates the feasibility of cell therapy with IL-10-DLI to promote fast immune reconstitution and tolerance after haplo-HSCT.

MATERIALS AND METHODS

PATIENTS AND TRANSPLANT PROCEDURES

Nineteen patients with high-risk hematopoietic malignancies, median age 39 years (range: 20–64), were enrolled from August 2000 to October 2009 in a single center, non-randomized prospective phase I–II clinical trial (ALT-TEN protocol) approved by the San Raffaele Scientific Institute’s Ethics Committee and the Italian Ministry of Health. The clinical trial was registered in the public cell therapy national database of the Italian Istituto Superiore di Sanità (registration number IS/11/6172/8309/8391). All patients signed informed consent. Enrolled patients were candidates for Sanità (registration number IS/11/6172/8309/8391). All patients were enrolled from August 2009 to January 2014.

The clinical trial was registered in the public cell therapy national database of the Italian Istituto Superiore di Sanità (registration number IS/11/6172/8309/8391). All patients enrolled in the trial were reported in Figure 1. Two (patients 8 and 17) of the 19 patients enrolled in the proof-of-concept ALT-TEN clinical trial were not transplanted because of relapse in the pre-conditioning phase and donor screening failure, respectively, thus they were excluded from the study because they did not undergo haplo-HSCT. Transplanted patients (n = 17) received a median of 12 × 10⁶ CD34+ cells/kg (range, 3.8–19.2), and 1 × 10⁶ CD3+ T cells/kg (range, 0.4–2.6). All but two transplanted patients did not receive IL-10-DLI because of graft failure (patient 12), or because they did not meet the eligibility criterion for early autologous reconstitution (patient 15 and patient 13, who also had no anergy). Therefore, five of the transplanted patients did not receive IL-10-DLI. Among them, two died because of infections (patients 10 and 12 at day 169 and 32 post-HSCT, respectively), two had disease recurrence (patients 13 and 15), and one (patient 19) received a second transplant from the same haploidentical donor because of graft failure.

IL-10-ENERGIZED T-CELL PRODUCTION AND PROTOCOL OF INFUSION

PBMC were collected by leukapheresis from the donors before G-CSF mobilization, and from the patients before the conditioning regimen. Cell manipulation was performed in GMP-validated conditions and cell product manufacture was approved by the Italian Ministry of Health. The method of cell production and the characterization of the cell product have been described in detail (25, 29). The donor-derived cell product was obtained after a mixed lymphocyte reaction (MLR) toward host-derived cells, in the presence of IL-10 (clinical-grade provided by Schering-Plough), for 10 days (MLR/10) (25). Host-derived stimulator cells were CD3-depleted PBMC. The cell product acquired host-specific hypo-responsiveness, called “anergy” and calculated as follows: [MLR – MLR/10 : MLR] × 100. Data obtained in preclinical studies showed that IL-10-energized cells do not change their phenotype as compared to the unmanipulated donor PBMC, do express very low activation markers such as CD25 and HLA-DR, are anergic toward the host alloAg in proliferation and have a decreased frequency of (antigen specific) cytotoxic CD8+ T cell precursors.

Percentages of anergy (≥67%, calculated as above and based on mean values –2 SD of percentage of anergy obtained from more than 30 donor pairs during the process of development) and cell viability (≥70%) were chosen as release criteria because considered to be essential for safety and efficacy of the infused cells. Cell
Table 1 | Outcome of the IL-10-DLI treated patients.

| Pt No. | Sex/age | Disease (status at haplo-HSCT) | Myeloid engraftment | IR | aGvHD (grade) | GvHD treatment duration (months) | Outcome (cause) | Overall survival
|--------|---------|-------------------------------|---------------------|----|---------------|--------------------------------|----------------|------------------
|        |         |                               | PMN                  | PLTs | Days from haplo-HSCT | Days from IL-10-DLI |                  | Days from haplo-HSCT |
| 1      | M/21    | HD (Advanced)                 | 18                   | 18  | no            | no                           | –               | –                | Death (infection)  | 43               |
| 2      | F/59    | sAML (Advanced)               | 13                   | ?   | no            | no                           | –               | –                | Death (relapse)    | 72               |
| 3      | M/31    | NHL (Advanced)                | 17                   | 26  | no            | no                           | –               | –                | Death (infection)  | 97               |
| 4      | M/39    | ALL (Advanced)                | 10                   | 10  | no            | no                           | –               | –                | Death (relapse)    | 35               |
| 5      | F/36    | AML (CR3)                     | 11                   | 11  | 18            | 24 (III)                     | 11              | –                | Death (infection)  | 355              |
| 6      | M/58    | NHL (Advanced)                | 23                   | 24  | 15            | 47 (III)                     | 39              | Alive/R          | 3017             |
| 7      | M/64    | AML (PIF)                     | 13                   | 15  | 28            | 34 (III)                     | 4               | Alive/R          | 2653             |
| 8      | M/20    | HD (Advanced)                 | 19                   | 18  | 102           | 99 (II)                      | 10              | Alive/R          | 2441             |
| 9      | F/22    | AML (CR2)                     | 17                   | 14  | 33            | 74 (II)                      | 1.5             | Alive/R          | 2339             |
| 11     | M/29    | HD (Advanced)                 | 12                   | 20  | no            | no                           | –               | –                | Death (infection)  | 69               |
| 12     | F/25    | HD (Advanced)                 | 22                   | 22  | no            | no                           | –               | –                | Death (graft rejection) | 44                 |
| 13     | M/39    | AML (CR1)                     | 17                   | 17  | no            | no                           | –               | –                | Death (graft rejection) | 43                 |

Pt No., patient number of enrollment. M, male; F, female. Diseases: HD, Hodgkin’s disease; sAML, secondary acute myeloid leukemia; NHL, non-Hodgkin lymphoma; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; MDS, myelodisplastic syndrome; Haplo-HSCT, hematopoietic stem cell transplantation from haploidentical donors. As “advanced status of diseases” are considered lymphoproliferative disorders in partial remission or with progressive disease and acute leukemia not in remission. CR, complete remission (CR1 first, CR2 second, CR3 third); PIF, primary induction failure; PMN, polymorphonucleated cells; PLTs, platelets; IR, Immune reconstitution; CD3⁺ T cells > 100/µl. IL-10-DLI, IL-10-anergized donor T cells. For patient 9, who received 2 IL-10-DLI, timing of immune reconstitution and acute GvHD onset is calculated from the first IL-10-DLI. aGVHD, acute graft-versus-host-disease.

IL-10-DLI, either fresh or frozen, were administered after sustained neutrophil engraftment and not before 1 month post-HSCT in order to exclude confounding overlapping events, such as acute GvHD due to contaminating donor T cells comprised within the graft and lytic effect of ATG toward the IL-10-DLI. All infusions were performed in the absence of Immunosuppression (IS). Eligibility criteria for IL-10-DLI were lack of immune reconstitution, defined by circulating CD4⁺ T cells ≤ 50/µl, no signs of GvHD, and absence of relapse. The protocol was designed for administration of escalating cell doses every three patients (10⁵, 3 × 10⁵, and 10⁶ CD3⁺ T cells/kg), or of the dose not considered toxic in at least 10 patients.

The primary endpoints of the cell therapy trial were safety defined as no acute adverse reactions, no bone marrow aplasia and lack of GvHD > grade II unresponsive to treatment. Secondary endpoints were immune reconstitution, defined as peripheral CD3⁺ T cells > 100/µl (value based on the time of effective immune reconstitution reported in previous trials of adoptive transfer performed at our Institute) (10), number of infectious episodes and restored in vitro immunological functions.

IMMUNOLOGICAL STUDIES

Routine tests, required during follow-up after haplo-HSCT, were performed at the HSR diagnostic laboratories (Laboraf), and included complete hematological evaluation, immunophenotyping (CD3, CD4, CD8, CD16, CD56, and CD19), monitoring of blood or tissue CMV and Epstein-Barr viral loads. Furthermore, immunophenotype analyses for surface detection of CD25 viability, phenotype, anergy, and proliferative capacity of each of the cell preparations infused in the patients showed no significant variability and are reported in the Table 2.

![Figure 1](https://example.com/image1.png)

**Figure 1** | Patients’ distribution and overall outcome of patients enrolled in ALT-TEN trial. IL-10-DLI: IL-10-anergized donor T cells; immune reconstituted patients: CD3⁺ T cells > 100/µl.
Table 2 | Immunophenotype and function of IL-10-DLI infused in patients of the ALT-TEN trial.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Treated (n = 12)</th>
<th>IR (n = 5)</th>
<th>Non-IR (n = 7)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
</tr>
<tr>
<td><strong>Immunophenotype (% of positive cells in the lymphocyte gate)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD2</td>
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<tr>
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<th>IR (n = 5)</th>
<th>Non-IR (n = 7)</th>
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<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
</tr>
<tr>
<td><strong>Anergy (%)</strong></td>
<td>76.8</td>
<td>7.7</td>
<td>80.4</td>
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<tr>
<td><strong>Proliferation (%)</strong></td>
<td>81.3</td>
<td>16.2</td>
<td>88.8</td>
</tr>
</tbody>
</table>

Cell composition and host antigen-specific unresponsiveness in secondary MLR, named anergy, and polyclonal response (to PMA/ionomycin, PMA/Ionom) of IL-10-DLI prior to infusion. Percentages are calculated as percentage of proliferative responses of MLR/10 versus MLR cell cultures. Mean values and standard deviations (SD) for the group of immune reconstituted (IR) and non-IR patients are reported. All cell products had vitality > 80%.

For in vitro functional assays total PBMC were plated at 10^6/well in 96-well plates in the presence of viral antigens: HSV-1 and -2 (2.5 µg/ml; Advanced Biotechnologies Inc., Columbia, USA) or CMV (lysat of infected human fibroblasts; diluted 1:30; kindly provided by Dr. Chiara Bonini, Laboratory of Experimental Hematology, HSR, Milan Italy). At day 4, cells were pulsed overnight with 1 µCi/well 3H-thymidine (Perkin Elmer, MA, USA). In parallel, cells were stimulated with coated anti-CD3 (10 µg/ml; OKT3; Orthoclone, Jansen-Cilag, Ratitan, NJ, USA) and soluble anti-CD28 (1 µg/ml; BD Pharmingen, San Diego, CA, USA) mAbs, PHA (1 µg/ml; Roche Diagnostics GmbH, Mannheim, Germany), and IL-2 (50 UI/ml; Chiron Italia, Milan, Italy) for 3 days. After stimulation, cells were pulsed for 16 h with 1 µCi/well 3H-thymidine. For IFN-γ ELISPORT analysis, multi-Screen HTS 96-well plates (Millipore, Billerica, MA, USA) were coated with captured anti-human IFN-γ mAb (IFNγ ELISpot KIT, ENELHIFNG, Thermo Scientific, Rockford, IL, USA) (30, 31). After saturation, thawed PBMC from patients and healthy donors were added in triplicate wells (3 × 10^5 cells/well) and were incubated for 18 h at 37°C in 5% CO2 in the presence of CMV infected cell lysate. Stimulation with PMA (10 ng/ml; Sigma, St Louis, MO, USA) plus Ionomycin (150 µg/ml; Sigma) was used as a positive control, and cells in medium alone were used as negative control. After washing, the second biotinylated anti-IFN-γ mAb (Thermo Scientific) was added, followed by Streptavidin–Alkaline Phosphatase conjugate (Roche) and then substrate (AEC Staining Kit, Sigma). After drying, spots were counted using an Elispot Reader (A. ELVIS GmbH, Hannover, Germany). The number of CMV-specific T cells, expressed as spot-forming cells per 1 × 10^6 PBMC, was calculated after the subtraction of negative control values.

T-CELL REPETOIRE ANALYSIS

TCR repertoire analysis (TcLandscape) was performed by TcLand Expression (Nantes, France), following the standardized and validated methods established in RISET [reprogramming the immune system for the establishment of tolerance (RISET)]. PBMC from patients’ blood, collected in EDTA tubes, were isolated by centrifugation over Lymphoprep Ficoll gradient (Axis-Shield) and frozen in EUROzol® reagent (Euroclone Spa Life Sciences Division, Milan, Italy) for RNA extraction. Total RNA was reverse-transcribed using an Invitrogen-Life Technologies cDNA synthesis kit (Roche Diagnostics). Complementary DNA was amplified by PCR using couples of primers specific for each Vβ genes. The amplification products were then elongated and electrophorized on a capillary sequencer (Applied Biosystems – Life Technologies 3730) to separate them according to their length. The complementarity-determining-region-3 (CDR3) profiles or spectra types obtained were transformed into mathematical distributions. In parallel, the level of Vβ family transcripts was measured by real-time quantitative PCR and normalized by a housekeeping gene (HPRT). Each CDR3 length distribution was then multiplied by the corresponding normalized Vβ transcript amounts and combined within the TcLandscape® representation (34). To explore the evolution of the TCR repertoire of a patient, we used the Principal Component Analysis (PCA) on the percentage of the Vβ family transcripts. This multivariant exploratory statistical technique is used to simplify complex data sets and to provide a “projection” of the data into a reduced, easily visualized space. In our context, (2A3, BD Biosciences, San Diego, CA, USA), CD45RA (M100, Biologend, San Diego, CA, USA), CD62L (DREG 56, BD Biosciences, San Diego, CA, USA), CD24 (ML5, BD Biosciences, San Diego, CA, USA), CD38 (HIT2, BD Biosciences, San Diego, CA, USA), CD49b (AK-7, Biologend, San Diego, CA, USA), LAG-3 (FAB2319P, R&D System) and intracytoplasmic detection of FOXP3 (259D, Biologend, San Diego, CA, USA), GZ-B (GB11, Caltag MedSystems, Buckingham, UK), and phycoerythrin (PE)-labeled anti-IL-10 (JESS-19F1, BD Biosciences), San Diego, CA, USA) were performed on freshly or frozen isolated PBMC at 37°C for 3 days. After stimulation, cells were pulsed for 16 h with 1 µCi/well 3H-thymidine. For IFN-γ ELISPORT analysis, multi-Screen HTS 96-well plates (Millipore, Billerica, MA, USA) were coated with captured anti-human IFN-γ mAb (IFNγ ELISpot KIT, ENELHIFNG, Thermo Scientific, Rockford, IL, USA) (30, 31). After saturation, thawed PBMC from patients and healthy donors were added in triplicate wells (3 × 10^5 cells/well) and were incubated for 18 h at 37°C in 5% CO2 in the presence of CMV infected cell lysate. Stimulation with PMA (10 ng/ml; Sigma, St Louis, MO, USA) plus Ionomycin (150 µg/ml; Sigma) was used as a positive control, and cells in medium alone were used as negative control. After washing, the second biotinylated anti-IFN-γ mAb (Thermo Scientific) was added, followed by Streptavidin–Alkaline Phosphatase conjugate (Roche) and then substrate (AEC Staining Kit, Sigma). After drying, spots were counted using an Elispot Reader (A. ELVIS GmbH, Hannover, Germany). The number of CMV-specific T cells, expressed as spot-forming cells per 1 × 10^6 PBMC, was calculated after the subtraction of negative control values.

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PCA allowed us to project the different time-points of each patient in a factorial space defined by axes, named components (C), which are linear combination of optimally weighted observed variables. The PAST software (Palstat: Statistics for Paleontologists and Paleobiologists. JS Whalley, PD Ryan 1995) was used for PCA and data representation.

**GENE-EXPRESSION ANALYSIS**

Microarray analysis was carried out by Miltenyi Biotec GmbH (Bergisch-Gladbach, Germany), according to the validated RISET methods. The data discussed in this publication have been deposited in NCBI’s Gene-Expression Omnibus (35) (GSE21885). Peripheral vein blood was drawn directly into PAXgene blood RNA tubes (QIagen, Crawley UK) and extracted using the PAXgene Blood RNA Kit, which includes DNase I treatment (QIagen). The quality and integrity of RNA were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was quantified by measuring A260 nm on an ND-1000 Spectrophotometer (Nanodrop Technologies). 0.5 μg total RNA was labeled using the Agilent Low RNA Input Linear Amp Kit (Agilent Technologies) as detailed in the “One-color microarray-based gene-expression analysis protocol” (version 5.5, part number G4140-90040). 0.6 μg Cy3-labeled fragmented cRNA was hybridized on the RISET 1.0 microarray platform according to the “One-color microarray-based gene expression analysis protocol” (version 5.5, part number G4140-90040). The last washing step was performed with acetonitrile for 30 s. RISET 1.0 is a custom Agilent 8 K microarray comprising 4,610 probes represented in triplicates that is dedicated to transplantation research and was designed based on published and unpublished data provided by RISET consortium partners. Probe design was optimized for the detection of multiple transcript variants of a gene, on optimized hybridization properties of the probes, and avoiding cross-hybridization. Fluorescence signals of the hybridized Agilent Microarrays were detected using Agilent’s Microarray Scanner System (Agilent Technologies, Inc.). The Agilent Feature Extraction Software (FES v. 10.2.1.3/10.5.1.1) was used to read out and process the microarray image files. To determine differential gene-expression, FES derived microarray data was further analyzed using the Rosetta Resolver gene-expression data analysis system (v. 7.2.2.0.0., Rosetta Inpharmatics LLC).

For time-course analysis, background-corrected signal intensity values were transformed to base two logarithms and quantile normalized. The open-source STEM (“Short Time-series Expression Miner v1.3.4”) software (36) was used with the default settings and the “No normalization/add 0” option. Expression profiles representing microarray probes with an increase or decrease expression level in the late-time samples (≥1 year after IL-10-DLI) were selected separately for each patient. An intersection of probes, identified for each patient, was applied for the generation of hierarchically clustered heat maps by using the MeV package (v. 4.5.1) (37). The lists of genes, with increased or decreased expression levels in the late-time samples of patients 7, 9, and 11, were analyzed for a significant enrichment of biological pathway annotation terms in comparison to the complete RISET 1.0 microarray configuration. Term-enrichment relative to the expected background distribution was scored using Fisher’s exact test with Benjamini-Hochberg correction. Annotations were derived from different sources, e.g., Gene Ontology (GO), signaling pathway membership, sequence motifs, chromosomal proximity, literature keywords, and cell-specific marker genes. No relevant terms were returned for the genes downregulated in the late-time samples (not shown). Gene-expression pattern of 166 time-course sensitive microarray probes were used for the generation of median inter-experiment correlation coefficients between patient and healthy control-derived samples, analyzed according to the time-span after starting IS and after its discontinuation. The microarray dataset including patients 7, 9, and 11 generated on the RISET 1.0 Agilent platform (GEO Series accession number GSE21885) was merged with the dataset obtained from the cohort of kidney transplant recipients studied by Indices of Tolerance (IOT) consortium (38) using the RISET 2.0 platform (GEO Series accession number GSE14655), considering only the 4610 reporters present on both platforms. Expression profiles of the 10 most discriminatory biomarkers of tolerance identified by the IOT study were extracted from the combined quantile normalized, log2-transformed dataset. From this data, pairwise Pearson correlation coefficients comparing the medians of each IOT patient (Tol-DF, tolerant drug-free; s-CNI stably immunosuppressed by calcineurin inhibitors (CNI); s-nCNI, stable under non-CNI; s-LP, stable under low dose prednisolone; HC, healthy controls) were calculated with each separate time-point from patients 7, 9, and 11.

**RESULTS**

**LONG-TERM SURVIVAL IN TRANSPLANTED PATIENTS TREATED WITH IL-10-DLI**

In a pilot study, 12 patients affected by high-risk/advanced stage hematologic malignancies and previously treated with multiple lines of chemotherapy, were transplanted with haplo-HSCT and received IL-10-DLI after myeloид engraftment (ALT-TEN trial). IL-10-DLI were infused at a median of 35 days post-HSCT (range, 28–64), when no spontaneous immune reconstitution was detectable in the periphery. Cells were infused in the absence of pharmacological IS. IL-10-DLI cell dose was 10^5 CD3^+ T cells/kg in all but one patient (patient 5), who received 3 × 10^5 CD3^+ T cells/kg. Four patients (patients 6, 7, 9, and 11), who received IL-10-DLI at the dose of 10^5 CD3^+ T cells/kg at a median of 40 days post-HSCT (range, 31–64), survived beyond day 100 post-HSCT and immune reconstituted (IR) at a median of 28 days post-IL-10-DLI (range, 15–102 days, Table 1). At present, these four long-term IR patients are IS-free with an optimal performance status at a median follow-up of 7.2 years (range 6.4–8.3). All four patients have a stable full donor chimerism. No disease relapse occurred in these reconstituted patients, despite the fact that two patients presented an advanced status of the disease at the time of transplant. Moderate acute GvHD was observed (grade II/III in patient 6 and grade II in patients 7, 9, and 11) starting at a median of

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60 days (range, 34–99) after IL-10-DLI. Acute GvHD completely resolved after conventional therapy administered for a relatively short period in patients 7, 9, and 11 (4, 10, and 1.5 months, respectively), whereas patient 6 required treatment for 39 months, and responded well to second-line therapy (Table 1).

Overall, IL-10-DLI was efficacious in 4 out of 12 patients, possibly because these four patients were free of events for a time period that was sufficient for immune reconstitution to occur. The remaining eight patients included three patients who died for infections early post-HSCT (day 43, 69, and 97 in patients 1, 14, and 3, respectively) and four patients who died because of relapse or lack of engraftment (patient 2, 4, 16, and 18) shortly after transplant. One patient (patient 5) received a higher dose of IL-10-DLI (3 × 10^10 CD3+ T cells/kg), which led to rapid immune reconstitution, but the patient developed severe GVHD unresponsive to treatment and died 1 year post-HSCT because of recurrent infections related to IS therapy (Figure 1; Table 1).

With the exception of the five IR patients described above, the overall survival of the patients treated with TCD haplo-HSCT and IL-10-DLI was comparable to that of patients receiving TCD haplo-HSCT without DLI. This negative outcome is due to the lack of immune reconstitution (not-IR) and is in line with that reported in high-risk patients treated with TCD haplo-HSCT without a complementary T-cell adoptive immunotherapy (“naked”) (1–3). None of the patients who failed to achieve immune reconstitution developed signs of GVHD.

IL-10-DLI cellular products received by the IR or not-IR patients were comparable in terms of cell composition (proportion of T, B, and NK-cells). Although not statistically different, the percentages of CD14+ and CD56+ were higher in IR patients as compared to non-IR patients (23.3 ± 14.6 vs. 12.5 ± 7.4% of CD14+ cells and 32.7 ± 17 vs. 20.5 ± 8.5% of CD56+ cells in IR and non-IR patients, respectively). Overall vitality, degree of host-specific-unresponsiveness and ability to proliferate in response to polyclonal stimulation of IL-10-DLI cell products in both IR and non-IR patients were not statistically significant (Table 2).

**IL-10-DLI PROMOTED FAST IMMUNE RECONSTITUTION AFTER TCD HAPLO-HSCT**

It is well established that after TCD haplo-HSCT complete functional restoration of T cells requires 6–9 months in pediatric recipients, and at least 1 year in adults (4, 5). In the four long-term surviving IL-10-DLI treated patients (patients 6, 7, 9, and 11), immune reconstitution occurred at a median of 30 days (range, 15–102) post-IL-10-DLI. At the time of immune reconstitution, the absolute number of CD3+ T cells was >250/µl (297, 394, 256, and 925 in patients 6, 7, 9, and 11 respectively), of CD3+CD4+ T cells was >150/µl (176, 163, 157, 294 in patients 6, 7, 9, and 11 respectively) and of CD3+CD8+ T cells was >100/µl (115, 231, 100, 631 in patients 6, 7, 9, and 11 respectively). Figure 2A shows the kinetics of natural killer (NK)-cell and T-cell recovery in peripheral blood. Peripheral NK cells repopulated shortly after IL-10-DLI (1–2 weeks). CD3+CD8+ T cells repopulated the peripheral blood before CD3+CD4+ T cells, leading to a persistent CD4/CD8 inverted ratio. The highest lymphocyte counts pre-GvHD were 1235, 1291, 500, and 1685 cells/µl, in patients 6, 7, 9, and 11 respectively. CD19+ B-cells were within normal limits (median, 98/µl; range, 72–251) at a median of 199 days (range, 150–276 days). These data show a faster peripheral lymphocytes repopulation in IL-10-DLI treated patients as compared to that reported in the literature for patients post-TCD haplo-HSCT (1).

In these latter patients, NK cell recovery occurred in 2–4 weeks, whereas T-cell immune reconstitution was delayed, with CD4+ T cells <100 and <200/µl 10 and 16 months after transplantation, respectively, and B-cell reconstitution required up to 2 years (6). In IL-10-DLI treated IR patient, peripheral T lymphocytes had an effector-memory phenotype (CD45RA−CD62L−), whereas naive T cells (CD45RA−CD62L+) were detected 1 year post-IL-10-DLI (data not shown). At long-term follow-up, total lymphocyte counts were within normal range (2100–3200 cells/µl) (Figure 2A) with naive/memory T cell subsets and transitional, naïve, and memory B-cell subsets all represented (Figures 2B,C).

**NORMAL TCR-VBETA REPERTOIRE IN IL-10-DLI TREATED PATIENTS**

The evolution of the TcLandscape demonstrated that in long-term follow-up IR patients, the TCR repertoire progressively became polyclonal and comparable to that of HC. The expression of the TCR-Vβ repertoires at different time-points during the follow-up is represented in Figure 3. Results in one representative patient (Figure 3A) show that the TCR repertoire in the IL-10-DLI cell product preinfusion was similar to that of the unmanipulated donor PBMC. We observed a flat landscape early post-HSCT, which indicates the absence of T cells. T-cell reconstitution started with olygoclonal T-cell expansion (red peaks in the landscape), corresponding to clinical signs of GVHD or infections (Figure 3A). Later on the TcLandscape gradually became polyclonal (green distributions) like the donor TcLandscape. The evolution of the TCR repertoire in the long-term follow-up patients (patients 7, 9, and 11) was also analyzed by PCA on each time-series of TcLandscapes performed (Figure 3B). PCA produces a map that summarizes the similarity of TCR-Vβ chain gene-expression considering recipient cells analyzed at all time-points, donor PBMC and IL-10-DLI TcLandscapes. It is noteworthy that donor PBMC and IL-10-DLI are close on the map indicating a similar TCR repertoire. The itinerary illustrates that the divergence between the donor T-cell repertoire and the repertoire of donor T cells post-haplo-HSCT decreases with time. The TCR of the recipient progressively resembles that of the donor, starting from a depleted T-cell landscape to a fully polyclonal T-cell repertoire.

**IMMUNE FUNCTIONS ARE RESTORED IN PATIENTS WITH LONG-TERM SURVIVAL**

In vitro proliferative responses to IL-2 were detectable early post-IL-10-DLI, consistent with the early reconstitution of NK cells. Proliferation in response to polyclonal stimuli [anti-CD3/CD28 monoclonal antibodies (mAb) or phytohemagglutinin (PHA)] and to viral antigens [CMV and Herpes-Simplex virus (HSV)] was detected between 6 and 12 months post-infusion, after immunosuppressive therapy withdrawal (Figure 4A). Interferon (IFN)-γ-producing CMV- and HSV-specific T cells (Figure 4B) were also detected by ELISPOT analysis, confirming the presence of viral reactive T cells in the peripheral blood of long-term IR patients. It should be noted that in historical controls in vitro T-cell function was still profoundly impaired 10 months after transplantation.
Overall, even if in a limited number of patients, immune reconstitution in IL-10-DLI treated patients was consistently faster and more efficient in comparison to historical controls, with polyclonal and Ag-specific functional responses normalized within the first year.

Consistently with what observed in vitro, in long-term IR patients (patients 6, 7, 9, and 11) bacterial and fungal infections and viral reactivations mainly occurred during pharmacological IS (Figure 2A). Overall, in the first 100 days post-HSCT, the estimated median of infectious episodes was 2.75 episodes/patient in the IL-10-DLI treated long-term IR group of patients, whereas in the groups of the untreated or IL-10-DLI treated but non-IR patients, the estimated median of infectious episodes was approximately 4 episodes/patient. Importantly, no mortality related to infections was observed in the four IL-10-DLI treated IR patients, whereas the IL-10-DLI patients who did not achieve immune reconstitution died for infections and relapse at a median of 72 days from haplo-HSCT.

IL-10-DLI IMMUNE RECONSTITUTED PATIENTS MAINTAIN HOST-AG-SPECIFIC UNRESPONSIVENESS AND DISPLAY MARKERS OF IL-10-MEDIATED TOLERANCE

Donor-derived PBMC isolated from IR patients post-IL-10-DLI did not proliferate in response to host-antigen-presenting cells (APCs), in contrast to PBMC collected pre-transplant. However, these cells displayed normal responses to third party allo-APCs in vitro (Figure 5A). These results demonstrate the host-specific unresponsiveness of donor T cells after IL-10-DLI and immune reconstitution.
It is possible that host-specific unresponsiveness is maintained by the Tr1 cells infused with the IL-10-DLI cell product or by Treg cells de novo induced during IR. To test this latter hypothesis, we investigated the presence of CD4+CD25+FOXP3+ natural T regulatory cells (nTregs) and Tr1 cells in IR patients. Percentages of nTregs were comparable in patients and in HC (average 5.2 and 4.4% in patients and HC; respectively, not shown), whereas a low proportion of cells with the Treg-specific DNA demethylated region (TSDR) of forkhead-box-protein-3 (FOXP3) was detected in patients' whole blood as compared to HC (average 0.49% in patients (range: 0.30–0.80%) and 1.4% in HC (range: 0.4–2.9%), in line with what previously described (39).

A significantly higher percentage of granzyme-B (GZ-B)+IL-10+ T cells ($P = 0.0006$) and of CD49b+ Lymphocyte Activation Gene 3 (LAG-3)^+ T cells ($P = 0.004$) was detected in the PBMC of IL-10-DLI treated patients compared to HC (Figures 5B,C). Importantly, cells expressing these biomarkers progressively increased during follow-up. This observation is consistent with the high mRNA expression of GZ-B, and LAG-3, between 6 months and 3 years post-TCD haplo-HSCT in IL-10-DLI treated patients (not shown).

In each patient, we compared the gene-expression profile at immune reconstitution, during GvHD and after immune-suppressive therapy discontinuation to that of three HC recruited in the “IOT” RISET-EU project (IOT^4). Consistently with the immune-reconstitution, the overall patients’ gene-expression

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FIGURE 4 | Immune functions in long-term surviving IL-10-DLI treated patients. (A) In vitro proliferative responses of PBMC to mitogens and specific antigens. Data are expressed as counts per minute (cpm) and are normalized for absolute number of lymphocytes: cpm obtained in response to addition of exogenous IL-2 are normalized to total lymphocyte counts. Cpm obtained in response to anti-CD3/CD28 (aCD3/CD28) monoclonal antibodies and other stimuli, phytohemagglutinin (PHA), and to the viral antigens Herpes-Simplex virus (HSV) and cytomegalovirus (CMV), are normalized to the number of CD3+ T cells. Asterisks indicate proliferative responses of haploidentical donor PBMC before transplant. Pt: patient; d: days; y: years. (B) Frequency of CMV- and HSV-specific T cells producing interferon (IFN)-γ in patients’ PBMC. Bars represent the number of IFN-γ producing T cells specific for CMV, HSV and PMA+ ionomycin (PMA/iono) in peripheral blood of patients (patients 6, 7, 9, and 11) tested 4, 3.5, 2.7, and 2.5 years after IL-10-DLI, respectively. Healthy controls (HC) were tested in parallel. Pt: patient.

profiles gradually approximated those of the HC, as visualized in a hierarchically clustered matrix view of gene-expression (Figure 6A) and calculated by the inter-experiment correlation coefficients (≥0.9). Of note, in the IL-10-DLI treated patients the gene-expression profile of the top 10 ranked genes recently identified as biomarkers of tolerance (38, 40) progressively became super-imposable to that of kidney transplanted recipients who did not reject the organ after IS withdrawal. Conversely, the gene-expression profile of the IL-10-DLI tolerant patients was different from that of transplanted patients under IS and from that of HC (Figure 6B). Notably, unlike in HC, in tolerant IL-10-DLI treated patients and tolerant kidney transplanted recipients, B-cell-related genes were highly expressed [Figure 6B and (38, 40)].

DISCUSSION
Patients with high-risk/advanced stage hematologic malignancies who received TCD haplo-HSCT, not followed by any kind of adoptive immunotherapy (“naked” haplo-HSCT) or followed by unmanipulated donor T-cell infusions, showed a very high rate of mortality because of infection and relapse or because of GvHD (3, 7). In the present study, 12 patients with high-risk/advance stage hematologic malignancies treated with TCD haplo-HSCT were infused with donor T cells anergized toward the host allo-Ags by IL-10. These IL-10-DLI contained both host-specific Tr1 cells and fully competent polyclonal T cells, which could down modulate host-reactivity and mediate effector responses, respectively (25). Of these 12, 4 patients had a positive outcome after TCD haplo-HSCT combined with IL-10-DLI, showing fast
immune reconstitution with restoration of normal T naïve and memory cell subsets, progressive increase in cells positive for the Tr1 biomarkers and a tolerance signature. These IR tolerant patients are long survivors, in good performance status and remain completely disease-free at 7.2 years follow-up (median).

Patients treated with IL-10-DLI who did not immune reconstitute displayed post-transplant courses that resemble that of “naked” transplants. It remains unclear why in these patients IL-10-DLI infusions were not sufficient to sustain immune reconstitution and to prevent infections and relapse. Pre-existing poor clinical conditions and advanced disease severity at the time of transplant could account for the lack of positive response to IL-10-DLI treatment (3, 41).

In the 4 long-term surviving patients, the infusion of IL-10-energized T cells shortly after engraftment of the myeloid compartment, correlated with an accelerated immune reconstitution with no relapse, only moderate and transient acute GvHD and no chronic GvHD. These data strongly support the notion that IL-10-DLI contributed to the overall success of the treatment. In line with this conclusion is the observation that once infused, the IL-10-DLI expanded, as demonstrated by the increase in NK-cells, CD8\(^+\), and CD4\(^+\) T cells, and by the progressive restoration of a polyclonal TCR-V\(_{\beta}\) repertoire. However, the infused donor-derived T cells specific for the host Ags remained unresponsive \textit{in vitro}. It has to be defined whether the control of host-reactivity is mediated by Tr1 cells present in the IL-10-DLI infusion, which survived long-term \textit{in vivo}, or is due to de novo generated Tr1 cells. Using a combination of novel Tr1 biomarkers, we showed a progressive expansion of GZ-B (42, 43)/IL-10 and CD49b/LAG-3-3 expressing CD4\(^+\) T cells in the peripheral blood. Importantly, the percentages of these cells were significantly higher in the IL-10-DLI long-term surviving patients [4, 5, 6] and 6 years after TCD haplo-HSCT for patient 6, 7, 9, and 11, respectively) compared to healthy subjects. CD49b and LAG-3 are bonafide biomarkers of Tr1 cells, which are consistently detected in tolerant patients with stable mixed chimerism after allogeneic HSCT (22). These data provide evidence of the long-term presence of Tr1 cells in fully immune competent tolerant patients 5 years after haplo-HSCT and IL-10-DLI treatment. Expansion of CD4\(^+\)CD25\(^+\)FOXP3\(^+\) T cells was not observed at any time in long-term IR IL-10-DLI treated patients suggesting that nTregs did not have a major role in the immune reconstitution and \textit{in vivo} immune modulation (44, 45).

Importantly, after HSCT, the gene-expression profile of IL-10-DLI treated patients was super-imposable to the tolerance signature of organ-transplanted patients with tolerant outcomes after IS withdrawal. Specifically, B-cell-related genes, associated with operational tolerance in stable kidney transplanted patients IS-free (38, 40) and in renal transplant recipient treated with regulatory macrophages (46) were observed in IL-10-DLI treated patients. This finding supports the hypothesis that IL-10-DLI

FIGURE 5 | Response to host allo-Ags and biomarkers of tolerance.

[A] Proliferative responses of donor-derived PBMC, toward host-monocytes before transplantation (pre). Proliferative responses of patient-derived PBMC from total PBMC of patients 6, 7, 9, and 11, tested 175 days, 2.8, 3, and 5 years; 180 days, 1.5, 2, 2.7, 4.2, 5, and 6 years; 190 days, 1 year, 3.5, 4, 4.2, and 6 years after IL-10-DLI, respectively, and of healthy controls (HC) (HC: \(n = 5\)). Mean ± SD of the percentages of IL-10- T cells of GzB\(^+\) T cells and of IL-10- GzB\(^+\) T cells in all time-points analyzed are shown. Differences were evaluated by means of the non-parametric Mann-Whitney U test. \(P<0.05\) were considered significant. (B) Intracytoplasmic granzyme-B (GZ-B) and IL-10 on gated CD4\(^+\) T cells from total PBMC of patients 6, 7, 9, and 11, tested 175 days, 2.8, 3, and 5 years; 180 days, 1.5, 2, 2.7, 4.2, 5, and 6 years; 190 days, 1 year, 3.5, 4, 4.2, and 6 years after IL-10-DLI, respectively, and of healthy controls (HC) (HC: \(n = 10\)). Mean ± SD of the percentages of IL10-T cells of GzB\(^+\) T cells and of IL-10- GzB\(^+\) T cells in all time-points years from haplo-HSCT. Differences were evaluated by means of the non-parametric Mann-Whitney U test. \(P<0.05\) were considered significant. cpm: counts per minutes of Tritiated Thymidine incorporation.
FIGURE 6 | Gene-expression profile analysis of three long-term follow-up patients. (A) Matrix view of gene-expression data (heat map) showing genes with significantly increased (violet vertical bar) and decreased (pink vertical bar) expression levels in samples evaluated at different time-points post-IL-10-DLI (as indicated in days or years) of patient 7, patient 9, and patient 11 compared to three samples from healthy controls (HC). Data represent quantiles normalized and median-centered signal intensity data in log 2 space. Red and green indicate upregulation and downregulation, respectively, relative to the reporter-wise median.

(B) Gene-expression analysis of the ten most discriminatory biomarkers of tolerance identified within the Indices of Tolerance (IOT) study in patients 7, 9, and 11. Pairwise Pearson correlation coefficients comparing the medians of expression in each IOT patient group (Tol-DF: tolerant drug-free; s-CNI: stably immunosuppressed by calcineurin inhibitors (CNI); s-nCNI: stable under non-CNI; s-LP: stable under low dose prednisolone; HC: healthy controls) with the expression in each separate time-point sample from patients 7, 9, and 11 are shown. The expression of the most discriminatory IOT biomarkers in IL-10-DLI treated patients progressively became super-imposable to that in Tol-DF patients and HC. The gray bars below each graph indicate immunosuppressive treatment.

treated patients have operational tolerance and provides evidence that the tolerance signature, once established, can be consistent across different clinical settings.

Results obtained in 4 haplo-HSCT transplanted patients who survived long-term after IL-10-DLI are promising especially considering that 2 of these patients were transplanted during disease relapse. However, the overall course of the trial highlights a number of limitations. In the majority of the IL-10-DLI treated patients relapse or infections occurred prior to or in time proximity with the infusion, when immunodeficiency was still severe. Therefore, the selection of patients may not have been optimal to reveal immune modulating effects of the Tr1 cells comprised in the IL-10-DLI cell product. In addition, it is possible that IL-10-DLI should have been administered earlier after transplantation, to allow parallel myeloid engraftment and immune reconstitution with better prevention of infections and relapse. Furthermore, the Tr1 cell frequency in the cell product was rather low as demonstrated in vitro by IL-10 ELIspot analysis (25). At the lowest effective dose, IL-10-DLI did not cause adverse effects and only moderate and transient GvHD. However, a threefold increase in cell dose provoked GvHD grade III-IV in one patient, suggesting that the ratio between effector cells and Tr1 cells was too high.

The variability in patient’s outcome was not due to differences in the anergic state of the infused IL-10-DLI since this parameter
was very consistent among all treated patients. However, it cannot be excluded that certain immune genetic characteristics of some patient/donor pairs, such as the combination of HLA-Ags mismatches, rendered them additionally susceptible to post-HSCT complications. In addition, the phenotype of infused IL-10-DLI cells was similar in IR patients vs. those who did not, although the former received an IL-10-DLI infusion that contained a higher, although not significant, proportion of CD56+ and CD14+ cells. The higher proportion of CD56+ cells in the IL-10-DLI may have led to earlier and better protection against infections, and possibly prevent disease relapse, after infusion, allowing T-cell expansion. Similarly, the higher proportion of CD14+ cells may reflect a higher number of APC with tolerogenic properties that could have favor, once in vivo, Tr1 cell differentiation and tolerance. Therefore, a possible contribution of NK cells and CD14+ cells in favoring faster immune reconstitution cannot be ruled out.

Early immune reconstitution post-HSCT has also been achieved with DLI genetically engineered to express the thymidine kinase suicide-gene (TK-DLI) (10), CD8-depleted DLI (47), CD25-depleted DLI (48), and CTLA4-Ig-treated BM T cells (49, 50). The incidence of severe GVHD remained high in all but the TK-DLI clinical trial, in which GVHD was controlled by acyclovir treatment, targeting the suicide-gene. Moreover, in these trials relapse occurred in a relevant proportion of patients (10). Freshly isolated or in vitro expanded Treg cells (13–15) have been infused in allo-HSCT or haplo-HSCT recipients to prevent GVHD. In these studies a reduction, but not abrogation, of GvHD was observed compared to historical controls. In addition, it should be noted that Treg cells were detected in the peripheral blood of treated patients only shortly after infusion (14). A major difference between the Treg cell trials and the trial with IL-10-DLI is that, in the former, a pool of polyclonal non-Ag-specific cells was administered, whereas we used a cell product containing adaptive in vitro primed Ag-specific Tr1 cells. A different approach to administer Ag-specific Tr1 cells has been reported in a cell therapy trial in patients affected with Chron’s disease. In this study OVA-specific IL-10 producing T cell clones were infused and subsequently re-challenged in vivo by feeding patients, with an egg-derived peptide. In this study safety and indirect evidences of clinical benefit were reported but no in vivo tolerance signature was observed (51).

Overall, the small proof-of-concept clinical trial described here demonstrates the feasibility of cell therapy with host-specific Tr1 cells and shows promising, although preliminary and uncontrolled, efficacy in 4 patients. Future phase I/II trials with larger cohorts of transplanted patients, homogeneous in terms of disease and treatment, are required to further prove the efficacy of this treatment. The present study opens the possibility of using Treg cells as adjuvant treatment also in organ transplantation, as planned for renal transplant recipient (The ONE study, European Union Funded Multinational Clinical Trial), and in other immune-mediated disorders, including inflammatory bowel diseases and type 1 diabetes.

AUTHOR CONTRIBUTIONS
Rosa Bacchetta and Maria-Grazia Roncarolo designed and coordinated the study, analysed the data and wrote the paper; Fabio Ciceri supervised the clinical study; Barbara Bacchetta collected and analyzed data and wrote the paper; Claudia Sartirana performed immunomonitoring; Silvia Gregori contributed to cell production, Tr1 studies and to manuscript editing; Maria T. Lupo Stanghellini, Raffaella Greco, Massimo Bernardi, Jacopo Peccatori contributed to clinical monitoring; Patrick Miqueu, did TCR studies; Stefan Tomiuk and Uwe Janssen did microarray analysis; Maria Hernandez-Fuentes contributed to gene-expression analysis; Elisabetta Zappone contributed to study design and initial clinical monitoring; Alessandro Ambrosi did statistical analysis; Monica Salomoni supervised cell production.

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