Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy

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ABSTRACT

Background
CD4+ regulatory T cells are a specialized subset of T cells that actively control immune responses. Several experimental protocols have been used to expand natural regulatory T cells and to generate adaptive type 1 regulatory T cells for regulatory T-cell-based therapies.

Design and Methods
The ability of exogenous recombinant human interleukin-10 to induce alloantigen-specific anergy in T cells was investigated and compared to that of interleukin-10 derived from tolerogenic dendritic cells, in mixed lymphocyte cultures. A detailed characterization of the effector functions of the resulting anergized T cells is reported.

Results
Interleukin-10, whether exogenous or derived from tolerogenic dendritic cells, induces a population of alloantigen-specific T cells (interleukin-10-anergized T cells) containing type 1 regulatory T cells, which are anergic and actively suppress alloantigen-specific effector T cells present within the mixed population. Interleukin-10-induced anergy is transforming growth factor-β independent, and is associated with a decreased frequency of alloantigen-specific cytotoxic T lymphocyte precursors, but interleukin-10-anergized T cells are still responsive to third-party, bacterial, and viral antigens. Tolerogenic dendritic cells are more powerful than exogenous interleukin-10 in generating type 1 regulatory T-cell precursors, and are also effective in the context of HLA-matched donors.

Conclusions
Based on these studies, we have developed an efficient and reproducible *in vitro* method to generate antigen-specific type 1 regulatory T-cell precursors starting from total peripheral blood cells with minimal cell manipulation and suitable for generating type 1 regulatory T cells for regulatory T-cell-based therapies.

Key words: regulatory T cells, immune responses, exogenous recombinant human IL-10.

Citation: Bacchetta R, Gregori S, Serafini G, Sartirana C, Schulz U, Zino E, Tomiuk S, Jansen U, Ponzoni M, Paties CT, Fleischhauer K, and Roncarolo MG. Molecular and functional characterization of allogantigen-specific anergic T cells suitable for cell therapy. Haematologica 2010;95(12):2134-2143. doi:10.3324/haematol.2010.025825

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RB and SG contributed equally to this paper.

Acknowledgments: the authors thank Eleonora Tresoldi for the excellent technical assistance she provided.

Funding: this work was supported by a grant from the Italian Telethon Foundation, the Italian Association for Cancer Research (Associazione Italiana per la Ricerca sul Cancro, AIRC) and EU FP6 integrated program (www.risetfp6.org).

Manuscript received on April 7, 2010. Revised version arrived on July 12, 2010. Manuscript accepted on August 3, 2010.

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The online version of this article had a Supplementary Appendix.

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Introduction

Regulatory T (Tr) cells are recognized as fundamental for the induction and maintenance of immune tolerance. Among CD4$^+$ Tr cells, the naturally occurring Tr cells and the adaptive type 1 regulatory T (Tr1) cells can be distinguished. Naturally occurring Tr (CD4$^+$CD25$^+$FOXP3$^+$) cells are generated in the thymus and express high levels of FOXP3, which is essential for their suppressive function.[1,2] Adaptive Tr1 cells are induced in the periphery in the presence of interleukin (IL)-10, secrete high levels of IL-10 in the absence of IL-4, and suppress T-cell responses through cytokine-dependent mechanisms.[3] Both Tr subsets have been demonstrated to be effective *in vivo* in mice[4,5] and in humans[6,7] in controlling immune responses not only in an alloantigen-specific context,[8] but also against a variety of other antigens including self-antigens, non-harmful antigens, and allergens.[3] Therefore, in the last decade much effort has been dedicated to establishing methods to isolate and expand or to induce Tr cells to be used as cell therapy to restore tolerance.[8,9]

IL-10 has a potent, broad spectrum of anti-inflammatory activities. Binding of IL-10 to its receptors, IL-10R1 and IL-10R2, activates the STAT3-mediated signaling that results in inhibition of different target genes.[10] The suppressive functions of IL-10 involve the inhibition of macrophages and dendritic cells (DC) with consequent down-regulation of the expression of major histocompatibility complex class II and co-stimulatory molecules,[11] and inhibition of pro-inflammatory cytokine production.[10] Since IL-10 inhibits the production of IL-12 by DC and macrophages, it has a key effect in suppressing Th1-mediated responses.[12] IL-10 can also exert positive effects on immune responses: it directly enhances IL-10 production by CD4$^+$ T cells,[12] it stimulates mast cells and B cells, and it acts as a growth factor for CD8$^+$ T cells and natural killer (NK) cells.[10]

The contribution of IL-10 in regulating T-cell-mediated responses has been linked to its role in inducing adaptive Tr1 cells and mediating their suppressive function. However, it is now evident that IL-10 can also be produced by effector T cells, not only by Th2 cells, originally described as the main source of IL-10, but also by Th1[13] and by Th17 cells.[14] We and others demonstrated that autocrine production of IL-10 by immature DC or by a specific population of tolerogenic DC, termed DC-10, is required for Tr1 cell differentiation *in vitro*.[15-17] IL-10 represents the critical factor involved in inducing Tr1 cells not only *in vitro*[4,18] but also *in vivo*.[19] High levels of IL-10 in plasma have, in fact, been correlated with tolerance,[20] and Tr1 cells can be isolated from peripheral blood of tolerant patients.[6,7]

Transforming growth factor-β (TGF-β) has potent modulatory effects on T cells since it inhibits IL-2 production[21] and prevents Th1 and Th2 differentiation.[22] Moreover, TGF-β can inhibit activation and maturation of monocytes and DC.[21] The production and functions of TGF-β and IL-10 are likely to be related, as IL-10 enhances TGF-β production and *vice versa*.[23] In the mouse, both TGF-β and IL-10 are necessary, but alone not sufficient, to induce antigen-specific T-cell hypo-responsiveness *in vitro* and to prevent graft-*versus*-host disease (GVHD) *in vivo* in a murine model of mismatched bone marrow transplantation.[24] IL-10/TGF-β-anergized T cells protect mice from GVHD when co-injected with naïve untreated cells, demonstrating that IL-10/TGF-β-anergized T cells contain the precursors of T cells with regulatory capacity which are able to suppress effector responses.

We previously demonstrated that IL-10 induces long-lasting anergy in both CD4$^+$ cells[4] and CD8$^+$ T cells,[25] and that IL-10-anergized cultures contain the precursors of Tr1 cells.[26] In this study we investigated the biological properties of anergic T cells obtained upon *in vitro* priming of T cells with allogeneic monocytes in the presence of exogenous recombinant human (rh) IL-10 (IL-10+monocytes) or IL-10-producing tolerogenic DC-10.

Design and Methods

Culture conditions and reagents

A detailed description of the culture conditions and reagents used in this study is available in the *Online Supplementary Appendix*.

Dendritic cell differentiation

DC-10 and mature (mDC) were generated as previously described.[15] Briefly, CD14$^+$ monocytes isolated as the adherent fraction of peripheral blood mononuclear cells (PBMC) were cultured with 10 ng/mL rhIL-4 (R&D Systems) and 100 ng/mL rhGM-CSF (R&D Systems) in the presence or absence of 10 ng/mL of rhIL-10 for 7 days. DC differentiated in the absence of IL-10 were matured on day 5 with lipopolysaccharide from *E. coli* (1 μg/mL, Sigma Chemicals, St Louis, MO, USA) for an additional 2 days. The purity and maturation state of DC were checked by flow cytometry to determine expression of CD1a, CD14, CD83 and HLA-DR.

Mixed lymphocyte cultures and proliferation assay

Human peripheral blood was obtained upon informed consent, from healthy donors, haploidentical donors, and HLA-matched unrelated donors, in accordance with local ethical committee approval (TIGET PERIBLOOD, and ALT-TEN) and with the Declaration of Helsinki. Healthy donors, who are not routinely HLA typed, were considered totally mismatched. Haploidentical and matched unrelated donor (MUD) pairs were serologically typed for class I HLA loci and HLA-DRB1 class II locus. MUD were also typed for HLA-DP1 and HLA-DQ1 class II loci. Full haplotype mismatching was defined as disparity at two or more HLA-A, B, C, and DRB1 loci. MUD pairs used in this study were classified according to the following typing: MUD pair #7, one mismatch in HLA-C (Cw02 *versus* Cw15); MUD pair #8, two mismatches in HLA-DPB1 (DPB1 0401 *versus* DPB1 0402, 0501); MUD pair #9, one mismatch in HLA-C (Cw03 *versus* Cw04). Post-Ficoll (Nycomed Amersham, Uppsala, Sweden) PBMC were used as responder cells and CD3-depleted cells or DC as stimulators. When CD3-depleted cells were used as stimulators, responder cells were plated at a 1:1 ratio: 5×10^5/well with the same number of stimulator cells in a final volume of 1 mL in 24-well plates (Costar, Cambridge, MA, USA), or 10^5/well in a final volume of 200 μL in round-bottomed 96-well plates, in the presence or absence of rhIL-10 (10 ng/mL). When DC were used as stimulators, responder cells were plated at a 10:1 ratio: 10^6/well PBMC with 10^5/well of DC (mDC or DC-10) in a final volume of 1 mL in 24-well plates (Costar), or 10^5/well PBMC with 10^4/well of DC in a final volume of 200 μL in round-bottomed 96-well plates. To evaluate secondary responses, primary cultures were carried out in 24-well plates for 10 days, in the presence or absence of IL-10. At day 7, half of the medium, with or without cytokines, was replaced by fresh medium. On day 10 cells were collected, washed and plated in 96-well plates with newly prepared stimulator cells (at a 1:1 ratio for CD3-depleted cells or 10:1 ratio for mDC), without the addition of cytokines. In some experiments rhTGF-β at 1 ng/mL, anti-IL-10R monoclonal antibody (3F9) or anti-TGF–β1,2,3 (1D11) at 10 μg/mL were used. After the indicated time, cells were either pulsed for 16 h with 1 μCi/well ^{3}H-thymidine or supernatants were collected for

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analysis of interferon (IFN)-γ production. To evaluate antigen-specific responses, after 10 days of culture, cells were harvested, washed and tested for proliferative responses or frequency of cytotoxic T-cell specific precursors (CTLp) to different stimuli as detailed in the *Online Supplementary Appendix*.

Skin explant assay

Alloantigen-primed cells, derived from MLR or MLR/10 cells performed with total PBMC as responders and as stimulators (2000 rad irradiated, 1:1 ratio) and cultured for 7 days in 25 cm^2 flasks, were collected and tested in a skin explant assay. The skin explant assay was performed as previously described.[27] Briefly, standard 6 mm punch skin biopsies were obtained from patients undergoing allogeneic haploidentical bone marrow transplantation. The punch skin biopsies were divided into eight to ten sections of approximately equal size and then co-cultured separately with the responder cells collected from MLR, MLR/10 or medium alone. After 3 days of co-culture, the skin sections were formalin-fixed, paraffin-embedded and stained with hematoxylin and eosin (HE). The histopathological evaluation of the skin sections was performed blindly and independently by two histopathologists (MP and CTP). The grading (grades I - IV) of graft-*versus*-host (GVH) reaction was performed according to the system described by Lerner *et al.*[28]

Enzyme-linked immunospot assay

After 10 days of culture, IL-10-secreting T cells in the anergized and control cultures were enumerated by enzyme-linked immunospot (ELISPOT) assay, according to the manufacturer's instructions (anti–IL-10 capture monoclonal antibody; clone M010, and anti–IL-10 detection monoclonal antibody, clone M011B, Endogen, Pierce, Rockford, USA). Spots were counted by a KS ELISPOT system (Zeiss Vision, Göttingen, Germany). The number of spots in control wells was subtracted from the spots in test samples.

RNA sampling and SuperAmplification

Total PBMC were activated with allogeneic monocytes in the absence (MLR) or in the presence of IL-10 (MLR/10) or with allogeneic DC-10 (MLR/DC-10) or mDC (MLR/mDC). After 10 days, cells were collected and were lysed using SuperAmp™ lysis buffer (Miltenyi Biotec GmbH) following the manufacturer's instructions and stored at -80°C. DNA microarray analysis of gene expression was performed at Miltenyi Biotec´s Genomics Service facility (Bergisch Gladbach, Germany), using 4x44K Agilent Whole Human Genome Oligo Microarrays (Agilent Technologies, Inc.). Further details are given in the *Online Supplementary Appendix*. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[29] and are accessible through GEO Series accession number GSE17493 (*http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17493*).

Statistical analysis

All analyses for statistically significant differences were performed with the Student's t test or non-parametric Wilcoxon's test. *P* values less than 0.05 were considered statistically significant.

Results

Interleukin-10 inhibits primary mixed lymphocyte reaction proliferation and induces T-cell anergy in haploidentical pairs

We previously described that exogenous IL-10 inhibits primary MLR proliferation of PBMC in response to mismatched monocytes (CD3-depleted cells).[30,31] We now show that using monocytes from haploidentical donors a similar inhibition can be observed, with a mean reduction in proliferation of 74±15% (n=9, *P*<0.0037) and of 79±14% (n=19, *P*<0.00002) in haploidentical and mismatched MLR/10, respectively, compared to MLR control cultures (Figure 1A).

T cells primed with haploidentical alloantigens in the presence of IL-10 became unresponsive to subsequent stimulation with the same alloantigen (with an average reduction in proliferation of 75±13%, n=10, *P*<0.0007, Figure 1B). Unresponsiveness was comparable to that observed in cultures of mismatched donors (81±15%, n=20, *P*<0.005). IL-10+monocytes, therefore, promoted anergy among pairs with different degrees of HLA disparity. An optimal effect was observed when the exogenous IL-10 was added at a concentration of 10 ng/mL on day 0 and the cultures were incubated for 10 days (*data not shown*).

Independently of the degree of HLA disparity of cultured cells, a significantly higher percentage of $CD14^+$ cells was observed after 10 days of culture with IL-10 compared to control cultures (7.3±4.4% *versus* 1.0±1.0% n=6, *P*=0.007, in MLR/10 *versus* MLR, respectively). The proportion of $CD4^+$ and $CD8^+$ T cells at the end of 10 days of culture with or without IL-10 was comparable. Mean percentages of 69±4% *versus* 61±8% for $CD4^+$ and of 21±4% *versus* 19±3% for $CD8^+$ were observed in IL-10-anergized *versus* control cultures. However, IL-10-anergized cultures showed a significant decrease in the expression of $CD25^+$ (mean values of 2±0.5% *versus* 22±14%, n=6, *P*<0.007 in IL-10-anergized and control cultures, respectively) and of $HLA\text{-}DR^+$ (mean values of 13±3% *versus* 38±1%, n=6, *P*<0.02 in IL-10-anergized and control cultures, respectively) on T cells compared to control cells, indicating a reduction in T-cell activation.

The human skin explant model has been shown to predict the risk of GVHD following allogeneic bone marrow transplantation in the setting of identical sibling transplantation.[27] Using the human skin explant assay we evaluated the ability of IL-10-anergized T cells to cause histopathological damage in haploidentical skins. Results were compared to damage of skin induced by cells obtained from control cultures (MLR) or by medium alone. IL-10-anergized cell cultures (MLR/10) caused significantly less skin damage than did control cells (Figure 1C). Grade 0 (undamaged skin) or grade I GVH reactions, defined by the occurrence of a mild vacuolization of the basal cell layer in the epidermis (arrows, Figure 1C), were observed in skin sections incubated with IL-10-anergized cell cultures or medium alone, whereas grade III skin GVH reaction lesions, consisting in detachment of the epidermis from the basal cell layer (arrows, Figure 1C), were detected in skin incubated with MLR cells. These results indicate that the severity of the histopathological damage in the human skin explant induced by IL-10-anergized cell cultures (grade 0-I) and the overall severity of the skin GVH reaction were significantly lower than that induced by control cells (grade II-III, *P*=0.008).

Anergy is associated with decreased frequency of alloantigen-specific cytotoxic T-cell-specific precursors and with a preserved capacity to respond to nominal and viral antigens

In unrelated allogeneic transplantations there is a direct correlation between the frequency of host-reactive CTLp in the graft and the incidence of acute GVHD *in vivo*. The overall alloantigen-specific CTLp frequency in the IL-10-

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anergized cultures was consistently lower than that in control cultures with mean percentages of reduction being 82±14% (n=10) in haploidentical pairs and 74±23% (n=20) in mismatched pairs (Figure 1D). The decrease in cytolytic activity induced by IL-10+monocytes was not only due to a decreased number of precursor cells, but also due to a qualitative defect, since the cytotoxic alloantigen-specific T cells present in IL-10-anergized cultures exerted a much lower lytic activity compared to those present in control cultures. The lower CTLp frequencies in haploidentical pairs (MLR >1/3000; MLR/10 >1/38000) compared to those observed in mismatched pairs (MLR 1/300; MLR/10 1>2300) are a direct consequence of the lower HLA disparity (≥2 HLA disparities) in haploidentical pairs *versus* total HLA disparity in totally mismatched pairs (Figure 1D). These observations show that IL-10+monocytes do not only inhibit proliferative response towards alloantigens but also interfere with the differentiation and expansion of allo-specific CTL. Moreover, these findings indicate that IL-10 exerts its inhibitory effects on both CD4⁺ and CD8⁺ T cells.

To determine whether the decreased antigen-specific response observed in IL-10-anergized cultures could be due to deletion of antigen-specific T cells, we studied the CD8⁺ T-cell response of HLA-A*0201 individuals against an immuno-dominant influenza matrix protein-derived peptide (MP.58-66), in the presence or absence of IL-10. Importantly, MP.58-66-specific CD8⁺ T cells in culture can be traced by Vβ17 expression, the dominant Vβ chain used by MP.58-66-specific T cells or by A2/MP.58-66 tetramers. After two rounds of antigenic stimulation, responder cells cultured without IL-10 (MPL) specifically lysed T2 cells pulsed with MP.58-66 peptide, whereas MP.58-66-specific cytotoxicity by cells cultured with IL-10 (MPL/IL-10) was strongly reduced (Figure 2A). A parallel reduction of peptide-specific IFN-γ secretion was observed (with a reduction of IFN-γ secretion of 76±13%, n=6, *P*=0.046, Figure 2B). In contrast, similar amounts of MP.58-66-specific T cells (Figure 2B and 2C) were observed in both MLP and MLP/10,

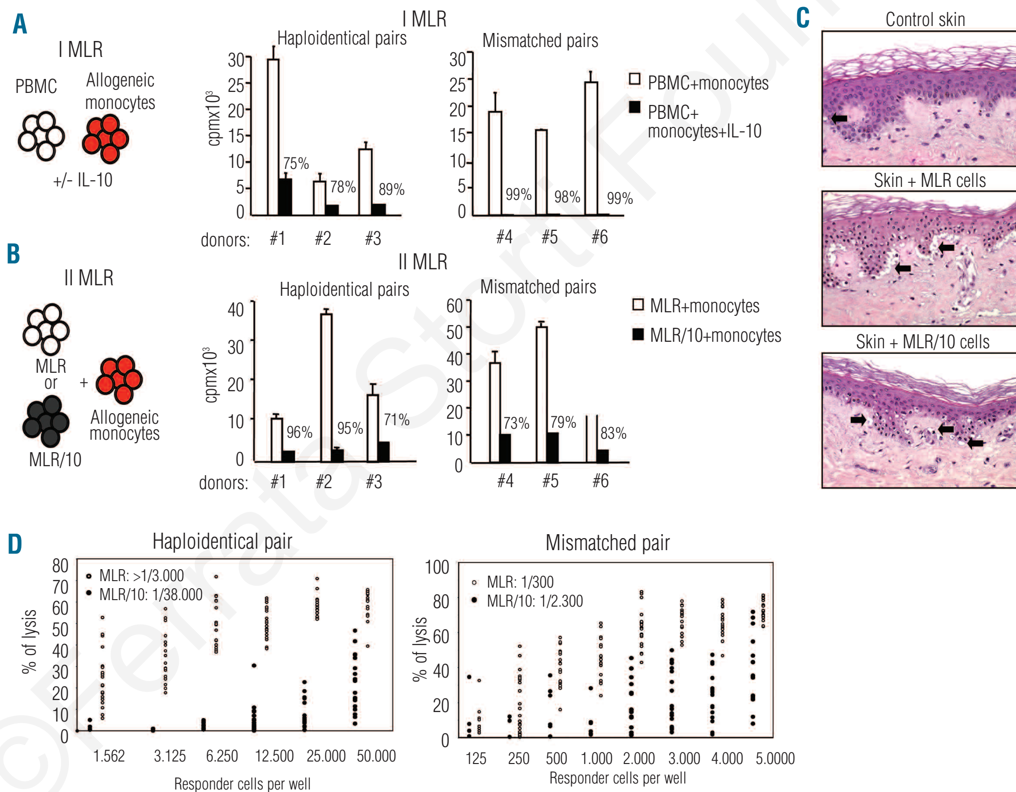

Figure 1. IL-10 inhibits primary allo-responses and induces anergy associated with a decreased frequency of antigen-specific CTL precursors. (A) PBMC were stimulated with allogeneic monocytes (CD3-depleted cells) in the absence (white columns, MLR) or presence of IL-10 (black columns, MLR/10). Proliferative responses were evaluated after 4 days of culture by adding ³H-thymidine for an additional 16 h. (B) PBMC were stimulated with allogeneic monocytes without (white columns, MLR) or with IL-10 (black columns, MLR/10) for 10 days. At the end of culture, T cells were collected and tested for their ability to respond to the same allogeneic monocytes used in the primary stimulation. Proliferative responses were evaluated after 48 h of culture by adding ³H-thymidine for an additional 16 h. Results represent the mean±SD of triplicate experiments. Three haploidentical donors (#1, #2, #3) defined as ≥2 HLA-A, B, C, DRB1 disparity, and three mismatched donors (#4, #5, #6) defined as totally mismatched pairs, are shown. Numbers indicate % of inhibition of proliferation in the primary stimulation (A) and % of anergy in the secondary stimulation (B), calculated as follows: 100-[(MLR cpm-MLR/10 cpm)/MLR*100]. C. PBMC were stimulated with CD3-depleted cells in the absence (MLR) or presence of IL-10 (MLR/10) for 10 days. After cultures T cells were incubated with haploidentical human skin biopsies. Hematoxylin and eosin staining of human skin biopsies incubated with medium (control skin), control cultures (skin + MLR cells), and IL-10-anergized cultures (skin + MLR/10 cells) are presented. One representative experiment out of three is shown. (D) PBMC were stimulated with allogeneic monocytes in the absence (MLR, black dots) or in the presence of IL-10 (MLR/10, white dots) for 10 days. After culture, T cells were collected and specific lysis of allogeneic stimulators used in the priming was evaluated. Cultures were tested in escalating concentrations in 20 replicates. The cut-off line of positive lysis was based on lytic activity against autologous cells ±3 SD. Representative data for one mismatched pair out of 20 tested and one haploidentical pair out of 10 tested are shown.

indicating that stimulation in the presence of IL-10 does not delete antigen-specific T cells, but rather renders them functionally inactive.

IL-10-anergized cell cultures, which were anergic towards alloantigens encountered during T-cell priming, showed a preserved ability to proliferate in response to tetanus toxoid (TT), *Candida albicans* (*C. albicans*), cytomegalovirus (CMV) or third party alloantigens (Figure 3A). In addition, IL-10-anergized cells proliferated vigorously in response to polyclonal stimuli such as TPA+ionomycin, indicating their preserved viability and proliferative potential (*data not shown*). Moreover, the frequency of CTLp against Epstein-Barr virus (EBV) was increased in IL-10-anergized cell cultures compared to control cultures (Figure 3B).

Overall, these results suggest that despite the fact that IL-10-anergized T cells acquire unresponsiveness towards a specific set of alloantigens, they retain, at the same time, the ability to mount an immune response to other antigens.

Transforming growth factor-β is dispensable for the induction of interleukin-10-anergized T cells

Exogenous TGF-β inhibited primary allogeneic stimulation with a mean reduction in proliferation of 51±14%, n=7, *P*=0.02, compared to the control MLR cells. This effect was significantly lower compared to that elicited by exogenous IL-10 (79±13%, n=7, *P*=0.02, reduction of proliferation compared to the control MLR cells). A mean percentage of inhibition of 95±4% (n=7, *P*=0.04) was obtained when both IL-10 and TGF-β were tested (*Online Supplementary Figure S1A*). Neutralization of endogenous TGF-β in primary MLR did not interfere with the strong inhibition of proliferation induced by exogenous IL-10 (79±19%, n=7, *P*=0.02 reduction compared to MLR; *Online Supplementary Figure S1A*). Conversely, neutralization of endogenous IL-10 resulted in a much lower inhibition of proliferation induced by exogenous TGF-β (37±24%, n=7, *P*=0.02 reduction compared to MLR; *Online Supplementary Figure S1A*). These results indicate that endogenous and exogenous IL-10 play a major role in inhibiting primary T-cell responses to alloantigens, whereas endogenous TGF-β can contribute to inhibiting T-cell proliferation but is dispensable.

Exogenous IL-10 or TGF-β alone inhibited secondary responses with an average inhibition of proliferation of 90±7% (n=5) and of 81±15% (n=5), respectively. Similarly, T cells primed in the presence of both IL-10 and TGF-β became hyporesponsive to re-stimulation with the same alloantigen, with an average inhibition of proliferation of 89±7% (n=5) (*Online Supplementary Figure S1B*). Blocking endogenous IL-10 during T-cell priming in the presence of TGF-β showed a reduced percentage of anergy (53±20%, n=5, reduction compared to MLR). Conversely, neutralization of endogenous TGF-β during T-cell priming in the pres-

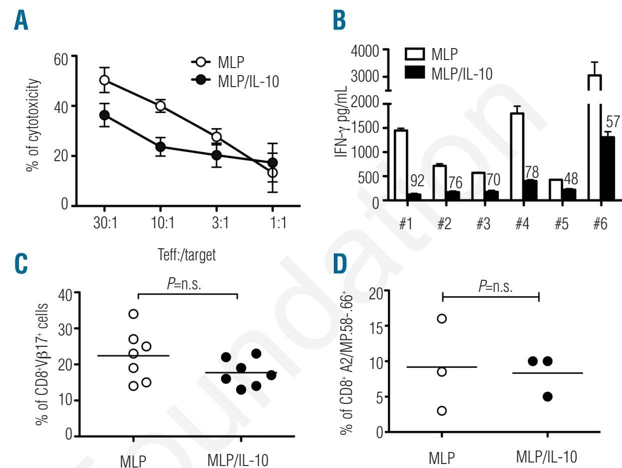

Figure 2. **IL-10 inhibits flu-specific T-cell responses without deleting antigen-specific effector T cells. (A) CD4⁻CD19⁻ PBMC isolated from a HLA-A2 typed healthy donor were stimulated with MP.58-66 peptide, in the absence (MLP) or presence of IL-10 (MLP/IL-10). After two rounds of stimulation, MLP and MLP/10 cells were re-challenged with T2 cells pulsed with MP.58-66 peptide and MP.58-66-specific lysis was measured. Lysis against unpulsed T2 targets was subtracted. One representative experiment out of three is shown. Results represent the mean±SD of triplicate experiments. (B) In parallel, MLP and MLP/10 cells were re-challenged with T2 cells pulsed with MP.58-66 peptide and IFN-γ was quantified in culture supernatants by ELISA. Results from each donor tested are shown. Results represent the mean±SD of triplicate experiments. The % inhibition of IFN-γ release in the presence of IL-10 relative to control is presented. (C) MLP and MLP/10 cells were stained with TCR-Vβ17-specific monoclonal antibody. Data are expressed as % of CD8⁺Vβ17⁺ T cells. Results from each of the seven donors tested are shown. (D) MLP and MLP/10 cells were tested in the tetramer-binding assay. Data are expressed as % of CD8⁺ T cells binding A2/MP.58-66 tetramers. Results from each of the three donors tested are shown.**

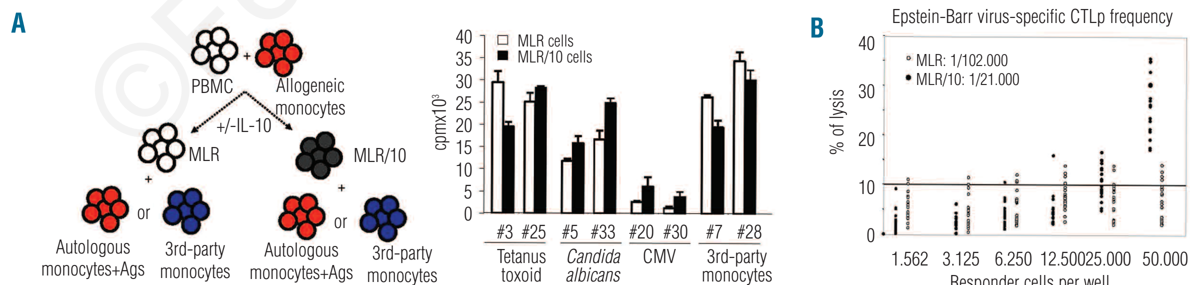

Figure 3. **IL-10-anergized cultures preserve the ability to proliferate towards nominal or third-party antigens. PBMC were stimulated with allogeneic monocytes without (MLR) or with IL-10 (MLR/10) for 10 days. (A) After culture, MLR (white bars) and MLR/10 (black bars) cells were stimulated with autologous monocytes in the presence of tetanus toxoid, *Candida albicans*, cytomegalovirus (CMV), and 3rd-party monocytes. Proliferative responses were evaluated after 4 days of culture by the addition of ³H-thymidine for an additional 16 h. Results are from one mismatched (defined as totally mismatched) and one haploidentical (defined as 2 HLA-A, B, C, DRB1 disparity) pair. Results represent the mean±SD of triplicate experiments. (B) Alternatively, the frequency of cytotoxic T cells specific for the EBV-LCL detected in MLR (black dots) and in MLR/10 (white dots) cultures was evaluated. The assay was performed in 20 replicates for escalating concentrations of cells. The cut-off line between positive and negative values was based on lytic activity obtained against autologous PHA-blasts. Results from one donor out of four tested are shown.**

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ence of IL-10 did not affect T-cell anergy induction (73±16%, n=5, reduction compared to MLR; *Online Supplementary Figure S1B*). It should be noted that cells anergized with TGF-β alone or in combination with IL-10 were completely unable to proliferate in response to *C. albicans* and to polyclonal stimuli such as TPA/ionomycin (*Online Supplementary Figure S1C,D*), indicating that in contrast to the murine cells,[24] exogenous TGF-β suppresses T-cell responses and abrogates the unique ability of IL-10 to generate antigen-specific anergic human T cells.

Tolerogenic dendritic cells inhibit primary mixed lymphocyte reaction proliferation and promote T-cell anergy

DC-10 have been demonstrated to be very efficient in priming naïve CD4+ T cells to become anergic alloantigen-specific Tr1 cells *in vitro*, since they are a source of endogenous IL-10 and express tolerogenic markers.[15] In contrast to fully mDC, DC-10 are CD14+ (73±28% *versus* 8±19%, mean±SD, n=10, in DC-10 and mDC, respectively) but CD1a- (2±2% *versus* 26±30%, mean±SD, n=10, in DC-10 and mDC, respectively), whereas like mDC, DC-10 have an activated phenotype being CD83+ (69±35% *versus* 63±28%, mean±SD, n=10, in DC-10 and mDC, respectively), and HLA-DR+ (90±18% *versus* 83±15%, mean±SD, n=10, in DC-10 and mDC, respectively). As expected, proliferative responses of PBMC induced by allogeneic DC-10 were consistently lower than those elicited by fully mDC. The inhibition of proliferation was observed without the addition of exogenous IL-10 and not only using mismatched or haploidentical cells as stimulators, but also using MUD antigen-presenting cells (Figure 4A). PBMC primed with mismatched DC-10 (MLR/DC-10) displayed a significantly lower proliferative response with a reduction in proliferation of 90±8% (n=14, P<0.00005), when compared to PBMC primed with mDC (MLR/mDC). Similarly, a significant inhibition of proliferation was observed in haploidentical and MUD pairs with a reduction of proliferation of 72±18% (n=5, P=0.02) and of 86±16% (n=10, P=0.0061), respectively, in comparison to PBMC primed with mDC. IFN-γ production by PBMC stimulated with DC-10 from mismatched, haploidentical, or MUD was significantly reduced when compared to production by PBMC primed with mDC (mean reductions of 93±14%, 95±7%, and 90±15% in mismatched, haploidentical, and MUD pairs, respectively, *data not shown*).

PBMC primed with DC-10 became unable to proliferate when restimulated with mDC from the same donor with a reduction in antigen-induced proliferation of 77±13% (n=18, P<0.0005) in mismatched pairs, of 78±8% (n=4, P=0.009) in haploidentical pairs, and of 78±14% (n=3, P=n.s.) in MUD pairs, in comparison to T cells primed with mDC (MLR/mDC) (Figure 4B). Comparable results were obtained when IFN-γ production by cultures re-challenged with mDC was measured (92±14%, n=14; 90±12%, n=3; and 99±2%, n=3 reduction compared to MLR, *data not shown*). Similarly to cultures with IL-10+monocytes, DC-10-anergized cultures, which were hypo-responsive towards alloantigen encountered during T-cell priming, were fully responsive to other antigens, such as TT, *C. albicans*, and third party antigen (*data not shown*).

Taken together these results indicate that DC-10 are efficient in promoting T-cell anergy independently from the degree of HLA disparities. Importantly, the use of DC as stimulators in cultures with T cells from MUD donors is

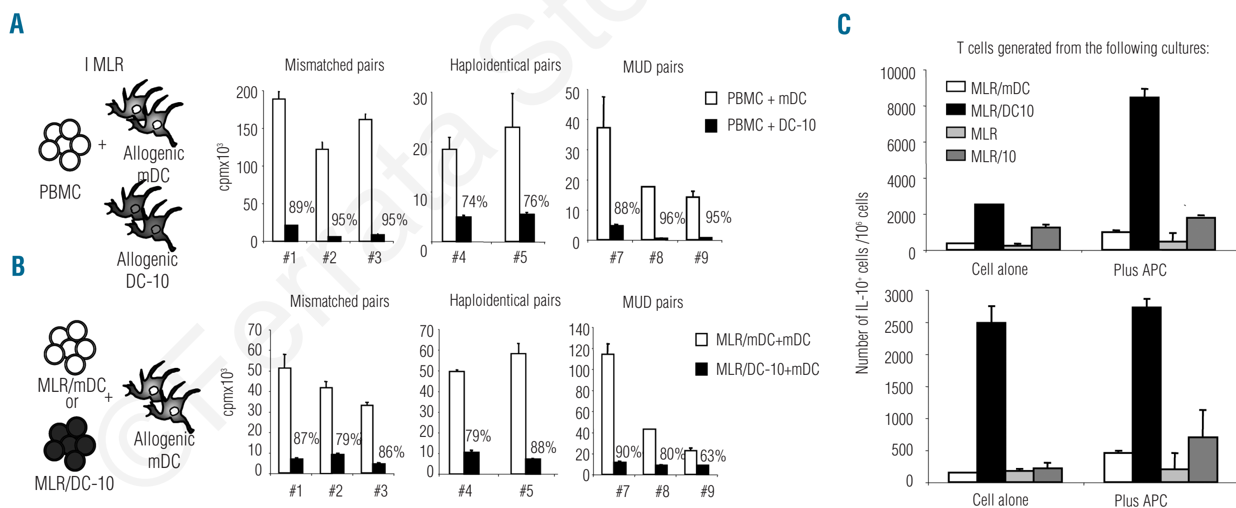

Figure 4. DC-10 induce T-cell hypo-responsiveness and T-cell anergy associated with a high frequency of IL-10-producing T cells. (A) PBMC were stimulated with allogeneic mDC (white columns, MLR/mDC) or with DC-10 (black columns, MLR/DC-10). Proliferative responses were evaluated after 4 days of culture by adding ³H-thymidine for an additional 16 h. Results represent the mean±SD of triplicate experiments. (B) PBMC were stimulated with allogeneic mDC (white columns, MLR/mDC) or with DC-10 (black columns, MLR/DC-10) for 10 days. At the end of culture, T cells were collected and restimulated with the mDC from the same donor used in the primary stimulation. Proliferative responses were evaluated after 48 h of culture, by adding ³H-thymidine for 16 h. Three mismatched donors (#1, #2, #3) (defined as totally mismatched), two haploidentical donors (#4, #5) (defined as ≥2 HLA-A, B, C, DRB1 disparity) and three MUD donors (#7; one mismatch HLA-Cw02 *versus* HLA-Cw15, #8; two mismatches HLA-DPB1 0401 *versus* HLA-DPB1 0402, 0501, #9; one mismatch HLA-Cw03 *versus* HLA-Cw04) pairs are shown. Results represent the mean±SD of triplicate experiments. Numbers indicate % inhibition of proliferation (A) and of anergy in terms of proliferation (B) calculated as follows: 100-[(MLR/mDC - MLR/DC-10) / MLR/mDC*100]. (C) PBMC were stimulated with allogeneic mDC (white columns, MLR/mDC), DC-10 (black columns, MLR/DC-10) or with CD3-depleted cells in the absence (bright gray columns, MLR) or presence of IL-10 (gray columns, MLR/10) for 10 days. At the end of culture, T cells were collected and restimulated with the mDC or CD3-depleted cells from the same donor used in the primary stimulation. Specific IL-10-producing T cells present in the cultures were counted by ELISPOT. Two different experiments are shown. Results represent the mean±SEM of triplicate experiments.

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necessary, since HLA-matched monocytes are unable to elicit a detectable allogeneic response *in vitro* (*data not shown*).

T-cell anergy induced by DC-10 was comparable to that induced by IL-10+monocytes in mismatched pairs (Figures 1 and 4). However, in haploidentical pairs in which IL-10+monocytes induced only limited T-cell anergy (inhibition of proliferation of 35%, and 36% when compared to that of control MLR), DC-10 were more effective in promoting anergy, with an inhibition of proliferation of 68% and 88% when compared to that of PBMC primed with mDC, respectively. DC-10 are, therefore, able to induce T-cell anergy in pairs in which IL-10+monocytes are not efficient. In line with this more potent effect, the frequency of IL-10-producing T cells in anergized cultures obtained with DC-10 was higher than that obtained in the presence of IL-10+monocytes (Figure 4C).

Gene expression profile of interleukin-10 and tolerogenic dendritic cell anergized T cells

The differential gene expression profile of anergized T cells obtained either in MLR/10 or MLR/DC-10 and in their respective controls (MLR and MLR/mDC) was analyzed by DNA microarray using a genome-wide approach with the aim of determining the gene signature of the cell product suitable for *in vivo* infusion. The whole unselected IL-10-anergized cell populations from three different donors stimulated with DC and monocytes from three distinct allogeneic donors were evaluated at the end of culture without further activation. Differential gene expression of at least 1.7-fold in at least two of the three biological replicates, with the third replicate showing the same trend, was considered significant. Overall, 44 genes were found to be significantly down-regulated and 66 genes were found to be significantly up-regulated in anergized cultures of MLR/10 and MLR/DC-10. To identify significant associations of IL-10-anergized cell-related genes with any specific molecular pathways screened by microarray, we performed annotation enrichment analyses.[32] The majority of the genes down-regulated in anergized cultures were found to have significant associations with cell proliferation pathways (cell cycle and mitosis) (24%), signal transduction pathways (36%), and modulation of cytoskeleton (21%) (Table 1). As summarized in Table 1, a large number of genes involved in signal transduction, in cell cycling, in cell division pathways (CENPF, CKAP2, NUSAP1),[33] and in T-cell activation such as IL2RA (CD25), and Lag-3 associated protein (CENPJ) were found to be down-regulated in anergized cultures. Conversely, the majority of the genes up-regulated in anergized cultures were found to have significant associations with pathways of immune responses (31%), wound repair (31%), inflammatory responses (26%), defense responses (30%), and cell migration (19%) (*Online Supplementary Table S1*). When analyzing the differentially expressed genes involved in immune response, special attention was given to identifying molecules linked to IL-10 and involved in modulating T-cell-mediated responses and T-cell migration. As summarized in *Online Supplementary Table S1*, in anergized cultures the scavenger receptors CD163, whose expression is induced by IL-10,[34] and SCARB2 were consistently up-regulated. Similarly, C-type lectins (CLEC4E and CLEC7A or Dectin-1) which are involved in stimulating anti-tumor and anti-microbial activity *in vivo*,[35] and chemokines (CCL2, CXCL1, and CXCL16) responsible for cell mobility, were over-expressed in anergized cultures.

A limited number of genes were up-regulated in MLR/10 *versus* MLR but down-regulated in MLR/DC-10 *versus* MLR/mDC (n=10), or were up-regulated in MLR/DC-10 *versus* MLR/mDC but down-regulated in MLR/10 *versus* MLR (n=6). These results indicate that anergized cultures obtained with IL-10+monocytes or with DC-10 displayed a number of common genes up- and down-regulated. However, some levels of differences in gene expression profile by using IL-10+monocytes or DC-10 were detectable, likely due to different kinetics of the T-cell response induced by monocytes or DC.

Discussion

In this study, we provide a comprehensive characterization of anergic T cells induced by exogenous rhIL-10 or IL-10-derived from tolerogenic DC-10. We previously established that IL-10 induces T-cell anergy when mismatched monocytes are used as stimulators.[31] We now demonstrate that IL-10-induced anergy is antigen-specific and is consistent among donors with different degrees of HLA disparities. IL-10-producing tolerogenic DC-10 represent a good alternative to exogenous rhIL-10 for promoting T-cell anergy in mismatched and haploidentical pairs. Importantly, DC-10 are required to promote T-cell anergy in MUD pairs in which HLA-disparity can be null, such as in matched pairs (8/8 alleles matched), or low such as in partially matched pairs (a single locus mismatch or multiple HLA mismatches).

IL-10-anergized T cells, although hypo-responsive towards the alloantigens used in primary stimulation, maintain their ability to respond to nominal antigens including bacterial antigens, such as TT and *C. albicans*, and viral antigens, such as EBV and CMV. IL-10-anergized T-cell cultures also have a reduced frequency of alloantigen-specific CTLp, whereas the frequency of EBV-specific CTLp is not reduced, but rather increased. Thus, exogenous IL-10 or tolerogenic DC-10-derived IL-10 promotes antigen-specific T-cell anergy without impairing the ability of the overall mixed population of cells to respond to other antigens. Notably, IL-10-anergized cells contain only a small proportion of *bona fide* IL-10-producing alloantigen-specific Tr1 cells (Figure 4C and[15]). Upon alloantigen stimulation the fraction of alloantigen-specific IL-10-producing Tr1 cells are activated via T-cell receptors (TCR), do not proliferate, but secrete high levels of IL-10 and TGF-β able to inhibit, directly and indirectly, the proliferation of the remaining alloantigen-specific effector T cells present in the mixed population. Conversely, upon activation with TT, *C. albicans*, CMV, or EBV, T cells, other than alloantigen-specific IL-10-producing Tr1 cells within the IL-10-anergized cultures, are activated *via* TCR and proliferate. As a consequence, IL-10-anergized cultures, either generated with rhIL-10 or with DC-10, display a TCR repertoire similar to that of the original unmanipulated responder cells (*data not shown*), confirming their multiple antigen-specificity. These findings suggest that IL-10-anergized T cells, being a mixed population of cells, may maintain the ability to mount an efficacious immune response against infectious agents when adoptively transferred into an immunodepressed host. Interestingly, by monitoring flu-specific $CD8^+$ T cells in IL-10-anergized cultures, we demonstrate that antigen-specific T cells are not eliminated but are impaired in their ability to produce IFN-γ, suggesting that IL-10, as a general mechanism, does not delete antigen-specific T cells but it renders them not functional.

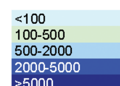
We prove the dispensable role of TGF-β in promoting IL-

10-induced anergy in human T cells. These results contrast with those obtained in the murine system, in which addition of TGF-β during polyclonal or antigen-specific T-cell priming in the presence of IL-10 resulted in a more profound and consistent hypo-responsiveness compared to that induced by IL-10 alone.[24] Both TGF-β and IL-10 are necessary, but alone not sufficient, to induce antigen-specific T-cell hypo-responsiveness in murine cells *in vitro* and to prevent GVHD *in vivo*.[24] In human cultures, TGF-β does not synergize with IL-10 in promoting T-cell anergy. Studies with neutralizing monoclonal antibodies demonstrated that IL-10 is fully capable of inducing anergy when TGF-β activity is eliminated. Although T cells primed in the presence of both IL-10 and TGF-β become hypo-responsive to second restimulation, they are no longer antigen-specific. The dispensable effect of TGF-β in promoting T-cell anergy in human cells is supported by our previous data demonstrating that TGF-β has no effect on the differentiation of Tr1 cells *in vitro*.[18] However, the mechanisms underlying the discrepancy between the role of TGF-β in murine and human T cells in the induction of antigen-specific hypo-responsiveness and, therefore, tolerance still remain to be elucidated.

The ability of IL-10 to induce anergy[25,26,31] is, at least partially, due to its effect of modulating T-cell activation. Analysis of IL-10-anergized cultures showed that, although the proportion of $CD4^+$ and $CD8^+$ T cells was comparable to

Table 1. Genes down-regulated in IL-10-anergized cultures in comparison to in control cultures.

		Donor #1				Donor #2				Donor #3				Pathways			
		MLR	MLR-10	MLR-mDC	MLR-DC10	MLR	MLR-10	MLR-mDC	MLR-DC10	MLR	MLR-10	MLR-mDC	MLR-DC10	T-cells (activated)	Cell cycle, mitosis	Apoptosis	Cytoskeleton
Signal transduction																	
IL2RA	interleukin 2 receptor, alpha (CD25)		-3.3		-5.5		-18.0		-2.0		-3.6		-1.7	■			
CKS1B	CDC28 protein kinase regulatory subunit 1B		-2.8		-8.4		-5.3		-11.6		-1.5		-17.5		■		
GPR174	G protein-coupled receptor 174		-2.0		-17.0		-13.8		-11.6		-1.9		-5.7				
MIB1	mindbomb homolog 1 (Drosophila)		-4.5		-5.7		-3.1		-1.8		-1.5		-2.2			■	
SHF	Src homology 2 domain containing F		-1.7		-2.7		-1.9		-4.9		-2.7		-2.9			■	
Associated with mitotic apparatus																	
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)		-2.5		-3.8		-5.8		-7.5		-8.1		-5.2		■		■
CENPF	centromere protein F, 350/400ka (mitosin)		-9.1		-30.4		-100.0		-1.8		-14.5		-11.6		■		■
CENPJ	centromere protein J		-11.7		-2.8		-13.8		-2.0		-9.5		-2.5		■		■
CEP152	centrosomal protein 152kDa		-2.8		-1.8		-10.4		-2.1		-1.6		-4.1				■
CKAP2	cytoskeleton associated protein 2		-5.2		-16.7		-2.0		-5.0		-1.9		-4.8		■		■
NUSAP1	nucleolar and spindle associated protein 1		-2.8		-15.6		-4.5		-4.8		-6.0		-3.9		■		■
SMG6	Smg-6 homolog, nonsense mediated mRNA decay factor (C. elegans)		-4.4		-1.6		-3.9		-3.6		-4.1		-6.9				
STAG2	stromal antigen 2		-2.0		-2.5		-2.2		-2.2		-1.7		-1.9		■		■
TTN	titin		-1.6		-3.2		-2.9		-1.8		-5.2		-1.7		■		■
Cytokines and chemokines																	
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10 (CD253)		-2.2		-1.7		-4.2		-2.7		-1.4		-3.0	■		■	
Enzymes																	
BCKDHB	branched chain keto acid dehydrogenase E1, beta polypeptide		-6.2		-1.8		-2.3		-4.8		-11.4		-7.7				
CASP2	caspase 2, apoptosis-related cysteine peptidase		-1.5		-2.4		-2.2		-2.0		-2.4		-1.8			■	
HELLS	helicase, lymphoid-specific		-2.1		-23.0		-5.5		-2.4		-2.1		-2.0				
MICAL3	microtubule associated monoxygenase, calponin and LIM domain containing 3		-2.5		-2.0		-2.0		-1.7		-2.6		-1.9				■
ZC3H12D	zinc finger CCCH-type containing 12D		-2.2		-2.3		-4.5		-2.4		-1.8		-1.6				
MCM3	minichromosome maintenance complex component 3		-2.9		-3.4		-3.7		-1.7		-7.6		-2.2		■		
WEE1	WEE1 homolog (S. pombe)		-1.9		-2.0		-1.8		-1.9		-2.1		-3.7		■		
MTMR7	myotubularin related protein 7		-2.3		-41.2		-2.9		-1.6		-100.0		-7.5				
Transcriptional regulation																	
AHRR	aryl-hydrocarbon receptor repressor		-1.5		-1.9		-3.0		-2.1		-4.3		-16.8				
INTS7	integrator complex subunit 7		-1.6		-5.0		-3.5		-2.0		-2.1		-2.5				
Hemoglobins																	
HBD	hemoglobin, delta		-1.8		-5.3		-100.0		-33.4		-4.6		-76.9				
HBG1	hemoglobin, gamma A		-16.9		-11.0		-100.0		-35.7		-2.7		-79.2				
HBG2	hemoglobin, gamma G		-8.2		-6.8		-47.4		-35.6		-2.2		-15.3				
Others																	
ARV1	ARV1 homolog (S. cerevisiae)		-1.8		-6.1		-2.5		-10.6		-4.2		-3.1				
KCNJ9	potassium inwardly-rectifying channel, subfamily J, member 9		-9.8		-3.3		-12.1		-6.2		-1.9		-1.7				
Unknown function																	
A_24_P488342	Unknown		-1.7		-4.2		-4.4		-3.0		-3.5		-1.8				
BI597240	Unknown		-3.4		-1.8		-6.9		-4.5		-2.7		-12.6				
C18orf1	chromosome 18 open reading frame 1		-2.0		-1.9		-5.7		-10.2		-1.8		-2.3				
C22orf40	chromosome 22 open reading frame 40		-1.6		-6.0		-2.4		-1.9		-2.2		-2.9				
CR610211	cDNA clone CS0DD002YI15		-6.1		-6.2		-2.9		-51.4		-4.7		-100.0				
DENND1B	DENN/MADD domain containing 1B		-3.9		-2.7		-2.9		-2.1		-3.6		-1.7				
KIAA0101	Unknown		-2.6		-1.9		-8.8		-2.5		-2.3		-3.1				
LYRM2	LYR motif containing 2		-3.5		-2.7		-3.0		-2.0		-6.3		-1.7				
NAG	neuroblastoma-amplified protein		-1.8		-2.9		-2.2		-7.7		-1.6		-6.0				
PACRGL	PARK2 co-regulated-like		-2.1		-2.6		-5.2		-1.9		-3.0		-1.5				
SAMD3	sterile alpha motif domain containing 3		-2.3		-1.7		-1.6		-1.9		-4.7		-11.3				
THC2499675	FLJ577090 (FLJ31159)		-1.7		-4.8		-8.0		-3.4		-51.8		-1.8				



Genes which were at least 1.7 fold down-regulated in at least two of the three biological replicates with the third replicate showing the same trend of both MLR/10 vs MLR and MLR/DC-10 versus MLR/mDC were selected and analyzed for a statistically significant enrichment of biological pathway annotation terms. Mid columns: the color range reflects the absolute level of gene expression. Differential expression between IL-10-anergized versus control cultures is represented in fold-change scale for each individual patient. Right hand columns: Assignment of the differentially expressed genes to significantly enriched biological pathway annotation terms. Term enrichment relative to the expected background distribution was scored using Fisher's exact test.

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that observed in control cultures, activation markers, including CD25 and HLA-DR, were significantly lower, indicating a direct inhibitory effect of IL-10 on T-cell activation. Accordingly, genome-wide expression profiling of the whole unselected IL-10-anergized cell populations obtained with both IL-10+monocytes and DC-10 revealed that genes involved in T-cell activation together with others involved in cell cycling, cell division, and mitosis were significantly down-regulated. mRNA expression of cytoskeleton-associated protein 2 (CKAP2), which is expressed only in actively dividing cells and not in cells in G0/G1 phase,[36] was consistently down-regulated in anergized cultures. This is in line with the observation that silencing this gene results in a significant reduction of cell proliferation.[36] Both centromer protein J (CENPJ) and centromere protein F (CENPF), which play important roles in cell division, centrosome functions, and in chromosome segregation,[33] were also consistently down-regulated in anergized cultures. The critical consequences of IL-10-induced anergy are, therefore, altered control of cell cycle activation and modified expression of cell division-associated molecules. These effects are similar to those observed in murine IL-10/TGF-β anergized cultures in which a selective defect in activation of cyclin-dependent kinases, controlling cell cycle progression, was shown.[37] Interestingly, gene expression profiling experiments demonstrated that a number of genes associated with IL-10 and with inhibition of immune-mediated T-cell responses were consistently up-regulated in all IL-10-anergized cultures independently of the anergizing conditions, Thus, IL-10 has a dual effect on T cells: it prevents T-cell activation and blocks cell cycle progression, and positively regulates a number of pathways involved in tissue repair and in inhibiting inflammatory responses such as C-type lectins and scavenger receptors (i.e. SCARB2 and CD163). Notably, the genome-wide approach is becoming an important tool for identifying biological signatures in autoimmune diseases.[38] Moreover, gene expression profiling of blood cells has been used to detect biomarkers of transplantation tolerance.[32] Using this approach we demonstrated that the overall gene signature of whole unselected IL-10-anergized cell populations, suitable for *in vivo* transfer, is one of anti-proliferation and anti-inflammation. These results are in line with and support the hypothesis that IL-10-anergized cultures, despite containing only a small proportion of alloantigen-specific Tr1 cells, modulate the gene expression profile of the surrounding effector T cells and thus their alloantigen-specific effector functions. These results are informative for the fate and function of the IL-10-anergized cells once infused *in vivo*.

The parallel induction of antigen-specificity and a gene signature of anti-proliferation and anti-inflammation render anergized cells, obtained in a short-term culture, particularly interesting for their potential clinical application. This consideration is especially relevant taking into account that a single stimulation of naïve $CD4^+$ T cells with allogeneic tolerogenic DC-10 is sufficient to induce a population of anergic T cells that contains up to 15% of allo-specific IL-10-producing Tr1 cells *in vitro* with suppressive activity.[15] Although IL-10+monocytes efficiently promoted T-cell hypo-responsiveness in PBMC independently from HLA disparities between responder and stimulator cells, the use of tolerogenic DC-10 represents an advantage compared to IL-10+monocytes in MUD pairs in which the HLA disparity is null or very low.

T-cell anergy has been demonstrated using different systems: typically it can be obtained by TCR ligation in the absence of full co-stimulation.[39] Blockade of the CD28/B7 pathway *in vitro* has been used to anergize donor-derived bone marrow cells towards haploidentical recipient cells prior to hematopoietic stem cell transplantation (HSCT).[40] In comparison with this latter approach IL-10-induced anergy results not only in decreased alloantigen-specific T-cell proliferation, but also in a reduction of alloantigen-specific CTLp frequency. Furthermore, IL-10-induced anergy is not due to a deletional mechanism but rather to induction of IL-10-producing cells which can give rise to alloantigen-specific Tr1 cells able to induce and sustain tolerance in the absence of immunosuppression.[6,15,26] These features offer a strong rationale for the use of IL-10-anergized T cells as cell therapy to improve immuno-reconstitution in immunocompromised hosts such as patients after allogeneic HSCT and to modulate responses to alloantigens and promote long-lasting tolerance. We hypothesize that, upon transfer *in vivo*, alloantigen-specific Tr1 cells are constantly activated within the allogeneic environment and secrete IL-10 and TGF-β locally, which suppress alloantigen-specific effector T cells and possibly promote the *de novo* induction of alloantigen-specific Tr1 cells *in vivo*. This autocrine loop would be critical for the induction and maintenance of alloantigen-specific tolerance *in vivo*. Indeed, *in vivo* transfer of alloantigen-specific IL-10-anergized T cells has been proven to be safe and feasible in patients with advanced hematologic malignancies undergoing T-cell-depleted haploidentical HSCT without immunosuppression (ALT-TEN trial, Bacchetta *et al.*, *unpublished data*). In addition, both methods described in the present study have been validated in GMP and could be applied to pairs with different degree of HLA disparities, including MUD pairs, and to different antigens. This represents an important and innovative finding that will broaden the clinical application of IL-10-anergized T cells.

In conclusion, our study shows the ability of exogenous IL-10 or tolerogenic DC-derived IL-10 to generate a population of antigen-specific anergic T cells to be used as cell therapy not only to inhibit GVHD after allogeneic HSCT, but also in other transplantation settings, or to re-establish tolerance in autoimmune or allergic diseases.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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