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HLA-G Level on Monocytoid Dendritic Cells Correlates with Regulatory T Cell Foxp3 Expression in Liver Transplant Tolerance

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Abstract

Background—Human leukocyte antigen (HLA)-G is a non-classical HLA class I molecule expressed as membrane-bound and soluble isoforms. Interaction of HLA-G with its receptor, immunoglobulin (Ig)-like transcript (ILT) 4 on dendritic cells (DC) down-regulates their T cell stimulatory ability.

Methods—We examined expression of HLA-G, ILT4, other immune regulatory molecules (inducible costimulator ligand and glucocorticoid-induced tumor necrosis factor-related receptor ligand), and the activation marker CMRF44 on circulating monocytoid (m) and plasmacytoid (p)DC by monoclonal antibody staining and flow cytometry. Three groups of stable liver transplant recipients, -operationally tolerant (TOL), prospective immunosuppressive drug weaning (PW) and maintenance immunosuppression (MI) were studied, together with healthy controls (HC). Serum HLA-G levels were measured by enzyme-linked immunosorbent assay.

Results—In TOL patients, mDC but not pDC expressed higher HLA-G than in MI patients or HC. In TOL patients, the incidence of CD4⁺CD25^{hi}CD127[−] regulatory T cells (Treg) and the intensity of Treg forkhead box p3 (Foxp3) expression were significantly higher than in the MI group. HLA-G expression on circulating mDC correlated significantly with that of Foxp3 in the TOL group. There was no correlation between immunosuppressive drug (tacrolimus) dose or

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Conflict of interest disclosure

The authors of this manuscript declare no conflicts of interest.

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trough level and HLA-G expression or Treg frequency or Foxp3 expression. The incidence of patients with circulating HLA-G levels >100ng/ml was highest in the TOL group, although statistical significance was not achieved.

Conclusions—Higher HLA-G expression on circulating mDC in TOL recipients compared with MI or HC, suggests a possible role of HLA-G in immune regulation possibly mediated by enhanced host Treg Foxp3 expression.

Keywords

HLA-G; dendritic cells; Foxp3; regulatory T cells; tolerance; liver transplantation

Operational tolerance (graft acceptance in an immunosuppressive drug-free environment) is achieved more commonly in liver (up to 20% of patients) than in other types of organ transplantation (1–4), especially in children (5, 6). Underlying mechanisms have not been elucidated. Moreover, identification of patients that can discontinue immunosuppression, without risk of rejection, has proved difficult, due to the absence of reliable biomarkers/ diagnostic tests that can discriminate the tolerant state (7). Recently, it has been reported (4) that transcriptional profiling of peripheral blood can identify adult liver graft recipients able to discontinue immune suppression, and that innate immune cells may play a major role in the maintenance of tolerance.

Dendritic cells (DC) are innate immune system cells that are also important in the induction and regulation of adaptive immunity (8). They have inherent tolerogenic properties (9, 10), including the ability to induce regulatory T cells (Treg), that have been associated with organ transplant tolerance (11, 12), including the spontaneous acceptance of liver allografts in mice (13). Moreover, the adoptive transfer of tolerogenic DC to experimental organ graft recipients can promote indefinite transplant survival (14–17). Several subsets of DC have been identified, principally conventional monocytoïd (m)DC, and also type-1 interferon (IFN)-producing plasmacytoïd (p)DC (18, 19), that may be specialized for modifying the strength, quality and duration of immune responses (20, 21). In previous reports, we have shown that the ratio of pDC:mDC is elevated significantly in operationally-tolerant pediatric liver transplant recipients (22, 23), suggesting that pDC may play a functional role in the tolerant state. There is also evidence that circulating levels of Treg are elevated in tolerant liver transplant patients compared with non-tolerant patients, or healthy individuals (6, 24, 25).

Immune regulatory molecules expressed by DC subsets, including B7-H1 (B7 homologue-1=programmed death ligand-1) (26, 27), inducible costimulatory ligand (ICOS-L) (28), glucocorticoid-induced tumor necrosis factor receptor-related ligand (GITRL) (29) and the non-classical HLA class I molecule HLA-G (30) and its receptor immunoglobulin-like transcript (ILT4) (31), have been shown to regulate T cell responses, including the induction of Treg. However, the expression of these molecules or the activation marker CMRF-44 (32) on DC has not been examined in relation to human organ transplant tolerance.

We examined the expression of these immune regulatory molecules on circulating DC subsets in operational pediatric liver transplant tolerance (TOL), patients undergoing prospective immunosuppressive weaning (PW), patients on maintenance immunosuppression (MI), and healthy controls (HC). Our data show that significantly elevated levels of HLA-G on mDC in TOL patients correlates with Foxp3 expression by CD4⁺CD25^{hi}CD127[−] Treg, suggesting that HLA-G expression on mDC may be of functional significance in immune regulation in the tolerant state.

RESULTS

Expression of HLA-G, ILT4, CMRF44, GITRL and ICOSL on circulating DC subsets

Flow cytometric analysis was first used to identify selected immune regulatory molecules on circulating mDC and pDC in 28 normal HC, as described in the Materials and Methods. Fig. 1A shows the gating strategy used to identify $\text{lin}^- \text{BDCA-2}^+$ pDC and $\text{lin}^- \text{BCDA-1}^+$ mDC, and the expression of HLA-G and ILT4 on these DC subsets. As shown in Fig. 1B, the incidence of mDC expressing HLA-G (but not ILT4) was significantly higher than that of pDC (HLA-G%: 57.62 ± 2.81 vs 27.19 ± 3.16 , $p < 0.0001$; ILT4%: 64.69 ± 3.87 vs 73.45 ± 2.81 , NS). Mean fluorescence intensity (MFI), however, for both HLA-G (33.25 ± 1.47 vs 55.46 ± 5.53 , $p < 0.0002$) and ILT4 (86.06 ± 12.83 vs 146.6 ± 21.27 , $p < 0.01$) was lower on mDC compared with pDC. As shown in Fig 1C, the frequency of circulating DC subsets positive for CMRF44 (pDC%: 1.62 ± 0.39 ; mDC%: 1.87 ± 0.54) and GITRL (pDC%: 3.88 ± 0.93 ; mDC%: 4.48 ± 0.80) was very low, with no significant difference between pDC and mDC. However, the frequency of ICOSL⁺ pDC was higher than ICOSL⁺ mDC (28.33 ± 3.82 vs 13.47 ± 2.77 , $p < 0.0034$). MFI, although low overall, was higher on pDC than on mDC for both CMRF44 (6.92 ± 0.23 vs 5.71 ± 0.38 , $p < 0.01$) and GITRL (10.64 ± 0.60 vs 7.93 ± 0.29 , $p < 0.0003$). Similarly, ICOSL MFI was higher on pDC than mDC (30.93 ± 2.02 vs 12.65 ± 0.74 , $p < 0.0001$) in HC (Fig 1C).

mDC in tolerant patients express elevated levels of HLA-G

We next compared the expression of HLA-G, ILT4, CMRF-44, GITRL and ICOSL on mDC and pDC in the 4 study groups. As shown in Fig. 2, expression of HLA-G (both % positive cells and MFI) was significantly higher on mDC in tolerant (TOL) patients compared with the maintenance immunosuppression (MI) (%: 68.8 ± 2.49 vs 58.89 ± 3.52 , $p < 0.05$; MFI: 42.95 ± 2.02 vs 35.37 ± 2.58 , $p < 0.05$) and HC groups (%: 68.8 ± 2.49 vs 57.62 ± 2.81 , $p < 0.05$; MFI: 42.95 ± 2.02 vs 33.25 ± 1.47 , $p < 0.05$) groups. No similar differences were observed for pDC. HLA-G expression on neither mDC nor pDC in the PW group differed from that in the other groups. ILT4 expression did not differ significantly between any of the 4 groups. Furthermore, we observed no differences in the low levels of expression of CMRF-44, GITRL or ICOS-L on either DC subset between the study groups (data not shown). HLA-G expression on mDC in the patient groups did not correlate with primary diagnosis, donor/recipient age at transplant, ABO typing, transplant type (whole or split liver), cold or warm ischemia time, induction therapy or liver function. In the patients studied, > 93% of PW and MI patients were under tacrolimus therapy (Table 1). In order to rule out a possible influence of tacrolimus in modulating the expression of HLA-G on circulating mDC, we analyzed the trough level and dose of tacrolimus in relation to HLA-G expression on mDC in individual PW and MI patients. As shown in Fig. 2C, no correlation between trough level/dose of tacrolimus and HLA-G expression on mDC (% positive cells or MFI) was observed.

Recently, it has been reported (33) that serum HLA-G levels are negatively correlated with the number of acute rejection (AR) episodes in liver transplantation. In our cohort of patients, we did not find any significant correlation between AR episodes (either no AR episodes versus 1 or more episodes, or 0–1 AR episodes versus 2 or more episodes) and HLA-G expression on mDC (MFI or % positive cells) in each group.

Serum levels of HLA-G do not differ significantly between tolerant, prospective weaning and maintenance immunosuppression patients

Given the elevated expression of HLA-G on circulating mDC in the TOL patients, we ascertained concomitant serum(s) HLA-G levels in each patient and control group by ELISA. Considerable variation was observed in sHLA-G levels (TOL: 63.3 ± 93.5 , PW: 92.3 ± 102.4 , MI: 67.1 ± 96.0 ng/ml), and no significant differences were detected between the

groups. Recently, it has been reported that in heart transplantation, only 13% of patients with sHLA-G levels >100 ng/ml suffered clinically significant acute cellular rejection compared with 63% of patients with sHLA-G <100 ng/ml (34). Interestingly, in our study population, a higher percentage of patients with sHLA-G levels >100 ng/ml was observed in the TOL group (9 out of 26: 34.6%) compared with PW patients (8 out of 28: 28.5%) and MI patients (6 out of 24: 25.0%) but these differences did not achieve statistical significance. As expected, in all HC, sHLA-G levels were very low or undetectable (2.1 ± 3.3 ng/ml). Although sHLA-G expression has been positively correlated with viral infection and neoplastic conditions (35), in our cohort of patients the frequency of Epstein Barr virus (EBV) infection and post-transplant lymphoproliferative disease (PTLD) was very low or absent (see Table 1). Therefore, we were not able to correlate the findings reported in our study with EBV infection or PTLD.

Tolerant patients exhibit significantly higher levels of circulating Treg than maintenance immunosuppression patients and controls

HLA-G has been reported to impair DC maturation (36) and to induce the development of tolerogenic mDC that can induce the differentiation of anergic and regulatory T cells (37, 38). We therefore compared the incidence of $CD4^+CD25^{hi}CD127^-$ cells (Fig. 3A), a phenotype that correlates with human $CD4^+$ Treg and their function (39) between the 4 study groups. For this analysis, we used a smaller, but not significantly different cohort of pediatric patients (18 patients for each group **Supplemental Table 1**), for which sufficient PBMC sample was available for analysis. As shown in Fig. 3B, the incidence of Treg was significantly higher in TOL than in MI patients ($1.59 \pm 0.19\%$ vs $0.86 \pm 0.12\%$, $p < 0.05$). Treg levels did not differ significantly between the TOL and PW groups ($1.59 \pm 0.19\%$ vs $1.56 \pm 0.20\%$, NS) or between TOL and HC ($1.59 \pm 0.19\%$ vs $0.93 \pm 0.09\%$, NS). Moreover, the incidence of Treg in the PW group did not differ significantly from that in HC ($1.56 \pm 0.20\%$ vs $0.93 \pm 0.09\%$, NS). The intensity of Foxp3 expression by $CD4^+CD25^{hi}CD127^-$ Treg was elevated significantly in the TOL group compared with the MI group ($162.7 \pm 11.22\%$ vs $104.1 \pm 13.68\%$, $p < 0.05$) but not compared with other groups (Fig. 3C). Treg (% $CD4^+$ cells and Foxp3 MFI) in the patient groups did not correlate with primary diagnosis, donor/recipient age at transplant, ABO typing, transplant type (whole or split liver), cold or warm ischemia time, induction therapy or liver function. As shown in Fig. 3D, no correlation was found between Treg (% $CD4^+$ cells or Foxp3 MFI) and trough/dose of tacrolimus in the PW and MI groups. There was also no correlation between rejection episodes and Treg in each patient group.

Elevated expression of HLA-G by mDC in tolerant patients correlates with enhanced levels of Treg Foxp3 expression

We next examined the relationship between HLA-G expression on mDC and the extent of Foxp3 expression in Treg in the 4 study groups. As shown in Fig. 4, there was a significant positive correlation between the intensity of HLA-G expression and that for Foxp3 in the TOL group ($p = 0.01$, $R = 0.39$) that was not observed in the other study groups (data not shown).

DISCUSSION

Both DC (12) and Treg (40) are thought to play important roles in the regulation of alloimmune responses and transplant outcome. Moreover, there is increasing evidence that these cells interact in the control of alloimmune reactivity (41). Thus, DC with tolerogenic potential can expand or induce Treg (17, 42–45), whereas Treg, in turn, can downregulate the immunostimulatory properties of DC and enhance their inherent tolerogenic function (46), resulting in feedback inhibition of effector T cell function and prolongation of graft

survival (47). Our understanding of these mechanisms is based largely on work conducted in vitro, or in experimental animal models, and there is little information regarding the phenotype of DC and their relation to Treg in human transplant recipients (48). Studies of the expression by DC of molecules associated with immune regulation in clinical transplantation may provide new insights into the role of these cells in relation to graft outcome.

Human DC express a variety of surface molecules that correlate with their maturation or activation status, and with their ability to induce or regulate immune responses. These include MHC gene products, the maturation marker CD83, the activation/differentiation-associated molecule CMRF-44 (32, 49), T cell co-stimulatory (eg CD80/86, ICOSL) and co-inhibitory molecules (eg B7-H1), and molecules that may be associated with the induction of Treg (eg HLA-G; GITRL). In this study, we did not observe any difference in the expression of specific maturation or costimulatory molecules or activation markers or coregulatory molecules on DC between groups of liver transplant recipients. We did however observe for the first time, that conventional mDC, but not pDC in tolerant liver transplant recipients expressed significantly higher levels of the non-classical MHC class I molecule HLA-G than those in patients on maintenance immunosuppression (MI). We consider it unlikely that this difference can be ascribed to use of immunosuppressive drugs (in the MI group) since these agents have been associated with enhanced HLA-G transcript and protein levels, both in vitro and in vivo (50–52). Moreover, we found no correlation between HLA-G expression on mDC and tacrolimus dosage or trough blood levels. HLA-G has been ascribed tolerogenic functions, both in pregnancy and transplantation (53, 54). In addition, we have found in this study that the enhanced expression of HLA-G by mDC correlates with concomitant, elevated expression of Foxp3 by blood-borne Treg, that, as reported herein and previously (6, 25), are elevated in tolerant pediatric liver graft recipients off all immunosuppression.

There is evidence that HLA-G plays an important role in regulating both the maturation and function of DC. Thus, interaction between HLA-G and its receptors leads to inhibition of DC maturation (55). Moreover, soluble HLA-G inhibits human DC-triggered allogeneic T cell proliferation (56), while HLA-G-expressing antigen-presenting cells induce CD4⁺ T cell anergy and differentiation of Treg (30). In HLA-G transgenic (tg) mice, DC maturation and T cell responses are compromised and skin allograft survival is prolonged significantly (36). Moreover, tg mice expressing human ILT4 only on DC and triggered by HLA-G exhibit long-term skin graft survival. Furthermore, HLA-G-modified DC from these tg mice promote long-term graft survival by mechanisms that include T cell anergy and induction of Treg (31). Taken together with our current observations, these findings suggest that elevated HLA-G on mDC in clinically tolerant liver transplant recipients may signify a functional role of HLA-G in achieving or maintaining the tolerant state. It would now be informative to determine the expression of HLA-G on mDC in sequential samples from liver graft recipients, to establish the relationship between these levels and progression towards the tolerant state. Such studies should include assessment of anti-donor T cell reactivity and its regulation that could not be performed in the present study due to the lack of donor cells.

The only previous study of HLA-G expression on both peripheral blood mononuclear cells and in serum of liver transplant patients led to the conclusion that the analyses performed could be beneficial in determining prognosis and response to treatment (51). However, in the present investigation, we detected a wide distribution of sHLA-G levels in the 3 patient groups, and there were no significant differences between these groups or between patients and HC. Nevertheless, in the TOL group, we found a higher incidence of patients (34.6%) with sHLA-G levels >100 ng/ml compared with the PW (28.5%) and MI (25.0%) groups, although the differences were not statistically significant. Recently, significantly higher

sHLA-G levels were reported in tolerant pediatric liver transplant patients, compared with patients who had experienced acute rejection episodes (33).

Transplant biopsies were not available in the present study, and therefore we were unable to determine intra-graft expression of HLA-G. Previously however, HLA-G expression in biliary epithelial cells and high serum concentrations of HLA-G have been associated with allograft acceptance in combined human liver-kidney transplantation (57, 58), whereas elevated expression of HLA-G in bronchial epithelium of human lung allograft recipients has been associated with graft functional stability (59). In view of these and the current findings, it would be of interest to evaluate expression of HLA-G both on parenchymal cells and non-parenchymal cells (in particular interstitial DC and other liver APCs) in relation to intra-graft and circulating Treg and human liver transplant outcome. In this regard, quantitative multiplex quantum dot immunostaining analysis of these variables in liver allograft biopsies sections (60) may be particularly informative.

In a previous cross-sectional analysis of liver allograft recipients (25), we observed that the ratio of costimulatory B7-H1:coinhibitory CD86 (B7-1) on circulating pDC, but not mDC, was higher in TOL compared with MI patients, and that this high ratio correlated with elevated Treg in operational liver transplant tolerance pDC undergo unique developmental programming and express a genetic profile that more closely resembles lymphoid than myeloid cell development (61), which may explain differences in regulation of surface molecule expression. The present findings concerning elevated expression of HLA-G on mDC (the predominant DC subset) in TOL patients provide further evidence of a possible functional relationship between the expression of immune regulatory molecules on DC subsets, the concomitant elevated frequency of Treg observed, and the state of operational liver transplant tolerance.

MATERIALS AND METHODS

Study population

Seventy-eight clinically stable, pediatric liver transplant recipients with normal graft function were eligible for study. All patients, and 28 normal HC, provided written informed consent in accordance with protocols approved by the local Institutional Review Board (protocol number: IRB010560). The demographics of this population, subdivided into four study groups, i.e. TOL: tolerant patients; PW: prospective weaning patients, MI: maintenance immunosuppression patients and HC are shown in Table 1. The mean age at transplantation for all patients was 5.3 ± 5.5 years (range 0.4–20.2 years), the mean time from transplant to testing was 8.9 ± 6.6 years (range 0.4–23.6 years), the mean age at testing was 14.2 ± 8.0 (range 1.6–27.9 years) and the mean time off all immunosuppressive therapy was 10.6 ± 4.5 years (range 3.5–16.9 years). Diagnoses at transplantation included cholestatic disease (n=40), metabolic disease (n=21), autoimmune disease (n=4), cryptogenic cirrhosis (n=2), and other diagnoses (n=10: comprising fulminant hepatic failure with unknown etiology, Budd-Chiari disease, total parental nutrition –induced liver failure, embryonal hepatoblastoma, and polycystic disease).

Patients off immunosuppression (TOL) or on low dose anti-rejection therapy undergoing prospective weaning (PW)

Twenty-six patients, off all immunosuppression (TOL), as described in Table 1, had been weaned off drugs by physician-directed protocol (n=18) as described (3), or emergently, for life-threatening infectious disease indications (Epstein-Barr virus [EBV], n=2; post-transplant lymphoproliferative disease [PTLD], n=2) or had self-weaned by noncompliance (n=3). Initial immunosuppression had consisted of azathioprine and prednisone (n=2),

cyclosporine and steroids (n=4), or tacrolimus and steroids (n=20). Briefly, the protocol used to achieve drug withdrawal was as follows: prednisone was withdrawn in 50% decrements monthly with corticotrophin stimulation testing to detect adrenal insufficiency if indicated clinically. Calcineurin inhibitors (primarily tacrolimus and, when applicable, cyclosporine) were then withdrawn at 1- to 2-month intervals by 10–25% of the baseline amount. Azathioprine, when present, was the final drug withdrawn. For patients who presented with acute complications of immunosuppression, such as EBV infection or overt PTLT, all immunosuppression was stopped immediately. Anti-rejection therapy was resumed when the presenting infection had resolved and with documentation of rejection on biopsy. Patients who did not subsequently require reinstitution of immunosuppression because of normal liver function and/or a severe episode of PTLT, were then included in the study group of patients off immunosuppression (TOL). Twenty-eight patients were undergoing prospective drug weaning (PW). All were on minimal immunosuppression, defined as monotherapy (93% of patients in the PW group were on tacrolimus monotherapy), with low (<5 ng/ml) or undetectable drug levels. Six months after blood sampling for the current study, the status of all of these patients remained unchanged.

Patients on maintenance immunosuppression (MI)

Twenty-four patients in this group had either failed drug withdrawal (n=10) or had never been weaned from immunosuppressive medications because of a concern for rejection and/or disease recurrence (i.e., history of autoimmune hepatitis) or previous rejection (n=14).

Healthy controls (HC)

For ethical reasons, blood was not drawn from normal healthy children and, as documented in previous studies (22, 23, 25), healthy adult volunteers of both sexes (n=28) served as controls.

Peripheral blood mononuclear cell (PBMC) isolation and cryopreservation

Peripheral venous blood samples were collected, and PBMC isolated, cryopreserved and recovered, as described in detail (25). We have shown previously (23) that results obtained staining cryopreserved PBMC do not differ significantly from those obtained using freshly-isolated cells.

DC subset analysis

As described previously (25), PBMC were stained on melting ice with a lineage (lin) monoclonal antibody (mAb) cocktail (anti-CD3, -CD14, -CD19, -CD20) and anti-HLA-DR. The cells were also stained at the same time with the DC subset-specific mAbs blood DC Ag (BDCA)-1 (CD1; clone AD5-8E7) and BDCA-2 (CD303; clone AC144) (both Miltenyi Biotec, Auburn, CA), as well as for HLA-G (87G) and the human DC differentiation/activation antigen CMRF-44 (CMRF-44) (32) (all from BD PharMingen, San Diego, CA), CD85d (ILT4) (Beckman Coulter, Brea, CA), glucocorticoid-induced tumor necrosis factor-related protein ligand (GITRL; eBioAITR-L) and inducible costimulator ligand (ICOS-L; MIH12) (eBioscience, San Diego, CA). DC subsets were identified as: mDC (lin⁻BDCA-1⁺BDCA-2⁻) and pDC (lin⁻BDCA-1⁻BDCA-2⁺; Fig. 1A). DC phenotype was further characterized by flow cytometric analysis (LSR II, BD Bioscience), gating on either the mDC or pDC population. Data were analyzed by using Flow-Jo or WinMDI software.

Treg analysis

PBMC were stained with fluorochrome-conjugated anti-CD4 (RPA-T4), and anti-CD25 (M-A251) from BD PharMingen, and with anti-CD3 (UCHT1), anti-CD127 (IL-3R; hIL-7R-M21) and anti-Foxp3 mAbs (PCH101) from eBioscience. Intracellular staining for Foxp3

was conducted after surface staining with anti-CD3, -CD4, -CD127 (IL-7R) and -CD25 mAbs, as recommended by the manufacturer (eBioscience). Treg were defined as CD4⁺CD127⁻CD25^{hi}Foxp3⁺ by flow cytometry, and the results expressed as % total CD4⁺ cells. Foxp3 expression was also expressed as mean fluorescence intensity (MFI).

Serum HLA-G quantitation

Serum HLA-G levels in groups of TOL, PW, and MI patients and in HC were determined by ELISA, using commercial kits from Biovendor Research and Diagnostic Products (Modrice, Czech Republic) and following the manufacturer's instructions.

Statistical analysis

Results are expressed as arithmetic means $\pm$ 95% confidence intervals. Statistical analysis was performed using Mann-Whitney *U* test or the Kruskal-Wallis test, where appropriate, followed by a post-hoc test (Dunn's test). Correlation analyses were performed using Spearman's correlation. For biomarker analysis on pDC and mDC in healthy controls, the significances of differences were determined using the unpaired Student's 't' test. 'P' values <0.05 were considered significant.

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Abbreviations

DC	dendritic cell
HC	healthy control
HLA	human leukocyte antigen
mDC	monocytoid dendritic cell
MI	maintenance immunosuppression
pDC	plasmacytoid dendritic cell
PW	prospective weaning
sHLA-G	serum human leukocyte antigen-G
TOL	tolerant
Treg	regulatory T cells

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FIGURE 1.

Expression of HLA-G, ILT4 and other regulatory molecules on circulating DC subsets in healthy controls. **A)** Flow analysis strategies used for the identification of DC subsets: PBMC were isolated, then stained for lineage (Lin) markers using anti-CD3, anti-CD14, anti-CD19 and anti-CD20 mAbs. pDC were discriminated using an anti-BDCA-2 mAb and mDC using an anti-BDCA-1 mAb. Molecules of interest on the DC subsets were analyzed as % positive cells and mean fluorescence intensity (MFI). **B)** Analysis of HLA-G and ILT4, and **C)** CMRF44, GITRL and ICOSL expression on circulating DC subsets in healthy controls. Mean values and 95% confidence intervals for the mDC and pDC are shown.

FIGURE 2.

HLA-G expression on mDC but not pDC is elevated in tolerant patients. **A)** HLA-G and **B)** ILT4 expression on circulating pDC and mDC in tolerant (TOL; 26), prospective weaning (PW; 28) and maintenance immunosuppression patients (MI; 24) and in healthy controls (HC; 28). Data are shown as % positive cells and mean fluorescence intensity (MFI). Mean values and 95% confidence intervals are shown. (* $p < 0.05$). **C)** Individual patient mDC HLA-G expression (% positive cells and MFI) and corresponding trough/dose of tacrolimus in the PW and MI groups.

FIGURE 3.

Regulatory T cells (Treg) and Foxp3 expression are elevated in tolerant patients. **A)** Representative gating strategy used for flow cytometric analysis. PBMC were stained using a combination of anti-CD3, -CD4, -CD127, -CD25 and anti-Foxp3 mAbs, as described in the Materials and Methods. Treg were defined as CD4⁺CD127⁻CD25^{hi}Foxp3⁺. Almost all CD4⁺CD127⁻CD25^{hi} cells ($\geq 94\%$) expressed Foxp3. **B)** Overall analysis of the frequency of circulating Treg and **C)** Foxp3 expression (MFI) in CD4⁺CD25^{hi}CD127⁻ T cells in the tolerant (TOL: 18), prospective weaning (PW: 18), maintenance immunosuppression (MI: 18) and healthy control groups (HC: 18). Mean values and 95% confidence intervals for the incidence of Treg and Foxp3 MFI are shown. (* $p < 0.05$). **D)** Individual patient Treg (% CD4⁺ cells and Foxp3 MFI) and corresponding trough/dose of tacrolimus in the PW and MI groups.

mDC - Tolerant

FIGURE 4.

Elevated expression of HLA-G on mDC in tolerant patients correlates with enhanced levels of Foxp3 expression. The data show the results of correlation analyses between HLA-G expression on circulating mDC (% HLA-G⁺ cells or HLA-G MFI) and the % CD4⁺CD127⁻CD25^{hi}Foxp3⁺ cells or Foxp3 MFI in tolerant patients (n=18).

Table 1

Demographics of study population

	TOL	PW	MI	HC
No. patients	26	28	24	28
Age at transplant, yr $\pm$ SD (range):	3.5 $\pm$ 5.6 (0.1–21.4)	4.8 $\pm$ 4.5 (0.2–16.2)	7.8 $\pm$ 6.1 (0.5–20.5)	NA
Time posttransplant, yr $\pm$ SD (range):	16.4 $\pm$ 5.5 (4.8–29.8)	6.9 $\pm$ 4.3 (0.9–19.1)	4.9 $\pm$ 4.4 (0.5–17.3)	NA
Age at study, yr $\pm$ SD (range):	20.1 $\pm$ 9.5 (7.4–51.2)	12.1 $\pm$ 5.0 (1.6–21.3)	12.5 $\pm$ 7.3 (4.2–23.9)	32.4 $\pm$ 4.3 (27.3–41.2)
Time off immunosuppression, yr $\pm$ SD (range):	10.6 $\pm$ 4.5 (3.5–16.9)	NA	NA	NA
Gender (M:F):	13:13	18:10	14:10	16:12
Recipient race				
Asian:	0	2	3	2
White:	25	25	20	24
Black:	1	1	1	2
Donor type				NA
Deceased:	25	23	19	
Living:	1	5	5	
Transplant type (% whole)	65%	62%	67%	NA
Cold ischemia time, min $\pm$ SD (range):	681.3 $\pm$ 442.9 (180–2253)	495.3 $\pm$ 265.3 (151–1087)	553.6 $\pm$ 202.7 (201–1011)	NA
Warm ischemia time, min $\pm$ SD (range):	45.5 $\pm$ 11.7 (29–60)	38.1 $\pm$ 9.3 (25–60)	37.7 $\pm$ 8.6 (25–55)	NA
Follow-up				NA
Time to wean from transplant, yr $\pm$ SD:	3.1 $\pm$ 3.1	4.6 $\pm$ 3.0	NA	
Time from wean to off, yr $\pm$ SD:	3.2 $\pm$ 3.8	NA	NA	
Diagnosis				NA
Cholestatic disease:	13	17	10	
Metabolic disease:	9	5	7	
Autoimmune disease:	2	0	2	
Cryptogenic cirrhosis:	0	1	1	
Other:	2	4	4	
Immunosuppression at study:				NA
Tacrolimus:	NA	26/28 (92.8%)	23/24 (95.8%)	
Cyclosporine:	NA	1/28	0/24	
Steroids:	NA	0/28	16/24	

	TOL	PW	MI	HC
Sirolimus:	NA	1/28	2/24	
MMF:	NA	0/28	2/24	
AZA:	NA	0/28	2/24	
Induction Therapy (%):	12.5%	28.0%	50.0%	NA
History of biopsy-proven EBV infection (%):	8.3%	0.0%	12.5%	NA
History of PTLD (%):	8.3%	0.0%	4.0%	NA
ALT, U/L±SD:	35.7±26.3	34.3±17.3	55.8±56.1	NA
AST, U/L±SD:	28.1±8.5	37.4±16.3	49.1±28.8	NA
Total bilirubin, mg/dl±SD:	0.8±0.5	0.5±0.3	0.6±0.4	NA
History of AR episodes	5/26 (20.0%)	7/28 (25.0%)	19/24 (79.0%)	NA
≥2 AR episodes	2/26 (7.7%)	3/28 (10.7%)	15/24 (62.5%)	NA
Time of last AR from study, yr±SD:	8.8±5.6	8.7±6.7	2.1±3.3	NA

TOL, tolerant; **PW**, prospective immunosuppression drug weaning; **MI**, maintenance immunosuppression; **HC**, healthy controls; **AR**, acute rejection; **AZA**, azathioprine; **MMF**, mycophenolate mofetil; EBV, Epstein-Barr virus; PTLD, posttransplant lymphoproliferative diseases.