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**Supplementary Material**

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Cytomegalovirus-Induced Expression of CD244 after Liver Transplantation Is Associated with CD8⁺ T Cell Hyporesponsiveness to Alloantigen

Emmy L. D. de Mare-Bredemeijer,* Xiao-lei Shi,* Shanta Mancham,* Rogier van Gent,* Marieke van der Heide-Mulder,* Renate de Boer,† Mirjam H. M. Heemskerk,‡ Jeroen de Jonge,‡ Luc J. W. van der Laan,‡ Herold J. Metselaar,* and Jaap Kwekkeboom*  

The chronic presence of viral Ags can induce T cell exhaustion, which is characterized by upregulation of coinhibitory receptors and loss of T cell function. We studied whether a similar phenomenon occurs after liver transplantation (LTx), when there is continuous exposure to alloantigen. Expression of coinhibitory receptors on circulating CD4⁺ and CD8⁺ T cells was analyzed longitudinally in 19 patients until 6 mo after LTx and cross-sectionally in 38 patients late (1–12 y) after LTx. Expression of the coinhibitory receptors CD160 and CD244 on circulating CD8⁺ T cells was already higher 6 mo after LTx compared with pre-LTx, and the elevated expression was sustained late after LTx, with CD244 showing the more prominent increase. The strongest upregulation of CD244 on circulating CD8⁺ T cells was observed in patients who experienced CMV infection after LTx. CMV infection also was associated with reduced CD8⁺ T cell proliferation and cytotoxic degranulation in response to alloantigen late after LTx. Purified CD244⁺CD8⁺ T cells from LTx patients showed lower proliferative responses to alloantigen, as well as to polyclonal stimulation, than did their CD244⁻ counterparts. In addition, the CD244⁺CD8⁺ T cell population contained the majority of CMV peptide–loaded MHC class I tetramer-binding cells. In conclusion, CMV infection after LTx, rather than persistence of alloantigen, induces the accumulation of dysfunctional CD244⁺CD8⁺ T cells in the circulation that persist long-term, resulting in reduced frequencies of circulating alloreactive CD8⁺ T cells. These results suggest that CMV infection restrains CD8⁺ T cell allosequences after LTx. The Journal of Immunology, 2015, 195: 1838–1848.

After liver transplantation (LTx), most patients need lifelong immunosuppression to prevent rejection of the allograft; however, some patients develop spontaneous immunological tolerance to their liver graft and can be completely withdrawn from all immunosuppression (1). This phenomenon occurs more frequently after LTx than in any other organ transplant setting (2), suggesting that the immunological alloreponse is skewed toward tolerance. Allograft rejection is primarily mediated by T cells of the recipient that respond to allologeneic donor Ags (3). In the transplantation setting, activation of allogeneic T cells via their TCR is triggered by specific recognition of donor-derived antigenic peptides presented by recipient MHC molecules or by direct interaction of the TCR with allogenic MHC molecules. However, T cells can also express costimulatory and coinhibitory receptors that affect the outcome of T cell responses (4). Hence, these receptors might also influence the outcome of T cell responses toward the allograft.

Expression of coinhibitory receptors is upregulated on T cells in patients with chronic viral infections, such as hepatitis C virus (HCV) and HIV, as well as in patients with cancer (5–12). High expression of coinhibitory receptors is associated with T cell dysfunction, or “exhaustion”; therefore, virus-specific or tumor-specific T cell responses are impaired in these patients. T cell exhaustion can be defined as a state of Ag-specific T cell dysfunction in response to chronic persistence of high antigenic load (5). Exhausted T cells have poor proliferative and effector function, show sustained expression of coinhibitory receptors, and their transcriptional state is distinct from that of functional effector or memory T cells (5). Several coinhibitory receptors were described to be important in inhibiting T cell responses; the most well-studied are programmed death 1 (PD1), lymphocyte-activation gene 3 (LAG3), T cell Ig mucin 3 (TIM3), CD160, and CD244. PD1 has two ligands: PD-L1 and PD-L2 (7, 13). PD-L1 is expressed on both hematopoietic cells (mainly on dendritic cells and macrophages) and nonhematopoietic cells (parenchymal cells of many organs, including liver; and endothelial cells) (7, 14). PD-L2 expression is restricted to dendritic cells and macrophages. Binding of PD1 to its ligand(s) negatively regulates T cell responses (13, 15). LAG3 is a protein closely related to CD4, and it mediates negative regulation of T cell functions through interactions with its ligand MHC class II, to which it binds with higher affinity than CD4 (16, 17). TIM3 inhibits CD8⁺ T cell responses by interaction with its ligand galectin-9 (8, 9). CD160 is a GPI-anchored receptor that inhibits T cell responses upon binding to its ligand, herpes virus entry mediator (HVE), which is expressed on both hematopoietic and nonhematopoietic cells (parenchymal cells) (18). CD244, also

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The online version of this article contains supplemental material.

Abbreviations used in this article: HBV, hepatitis B virus; HCV, hepatitis C virus; HVEM, herpes virus entry mediator; LAG3, lymphocyte-activation gene 3; LTx, liver transplantation; PD1, programmed death 1; PF, precursor frequency; Tem, central memory T cell; Temra, effector memory T cell; Temrma, terminally differentiated T cell; TIM3, T cell Ig mucin 3; Tr, naive T cell.
called 2B4, can mediate both activating and inhibitory signals upon binding with its ligand, CD48. High levels of CD244 expression on T cells were found to be associated with inhibitory receptor function (10, 14, 19).

After organ transplantation, the numbers of circulating T cells that react to donor alloantigens decrease over time in a majority of patients (20–22). The mechanism underlying this phenomenon is unclear. Whether chronic stimulation by the persistence of a high alloantigenic load induces upregulation of coinhibitory receptors and exhaustion of donor-specific T cells, as observed during chronic viral infections, is unknown. However, various experimental animal studies showed enhanced rejection and/or decreased graft survival after blockade of coinhibitory receptors in organ-transplanted mice. This implies that coinhibitory receptors are involved in suppressing alloagraft rejection in mice (23–26). However, the role of T cell exhaustion and coinhibitory receptor–ligand interactions in human solid organ transplantation have not been widely studied (25).

The aim of this study was to determine whether the expression of coinhibitory receptors on circulating T cells is upregulated after LTx in humans, to analyze which clinical factors influence such upregulation, and to assess whether coinhibitory receptors impair allogeneic T cell responses after LTx. We hypothesized that long-term persistence of a high antigenic load after LTx may induce the exhaustion of allogeneic T cells, which is characterized by upregulation of coinhibitory receptors and hyporesponsiveness of CD8+ T cells to alloantigens.

Materials and Methods

Study design and patients

Heparinized blood was collected at 1 and 6 mo after transplantation from 19 primary liver transplant recipients who were transplanted at the Erasmus MC-University Medical Center (early post-LTx cohort). In addition, blood was collected during a single regular visit at the outpatient clinic from 38 stable primary liver transplant recipients 1 to 12 y after transplantation in the Erasmus MC-University Medical Center (late post-LTx cohort). Multigraft transplantation patients were excluded. The occurrence of CMV infection (either primary infection or reactivation) after transplantation was determined from cryopreserved splenocytes, isolated using Ficoll-Hypaque density gradient centrifugation. Cryopreserved PBMCs collected from patients and healthy individuals were isolated using Ficoll-Hypaque density gradient centrifugation. Cryopreserved PBMCs were thawed and labeled with 0.5 μM CFSE. CFSE-labeled PBMCs were stained with anti-CD3–PerCP-Cy5.5, anti-CD4–PerCP, and anti-CD8–eFluor 450 (RPA-T8; eBioscience) (27). PBMCs and expanded B cells were collected and used for baseline measurements. Cryopreserved splenocytes, isolated using Ficoll-Hypaque density gradient centrifugation. Cryopreserved PBMCs collected from patients and healthy individuals were isolated using Ficoll-Hypaque density gradient centrifugation.

Cell culture

PBMCs from patients and healthy individuals were isolated using Ficoll-Hypaque density gradient centrifugation. Cryopreserved PBMCs collected from patients and healthy individuals were isolated using Ficoll-Hypaque density gradient centrifugation. Cryopreserved PBMCs were stained with anti-CD3–PerCP-Cy5.5 (UCHT1; BD Biosciences), anti-CD4–PerCP, and anti-CD8–eFluor 450 (RPA-T8; eBioscience) (27). PBMCs and expanded B cells were collected and used for baseline measurements. Cryopreserved splenocytes, isolated using Ficoll-Hypaque density gradient centrifugation. Cryopreserved PBMCs collected from patients and healthy individuals were isolated using Ficoll-Hypaque density gradient centrifugation.

Flow cytometry

Flow cytometry was performed to determine T cell subsets and coinhibitory receptor expression. For analysis of CD4+ and CD8+ T cells, isolated PBMCs were stained with anti-CD3–Horizon V500 (UCHT1; BD Biosciences, Erembodegem, Belgium), anti-CD4–allophycocyanin–H7 (SK3; BD Biosciences), and anti-CD8–eFluor 450 (RPA-T8; eBioscience, Vienna, Austria). To distinguish naive and memory T cell subsets, cells were stained with anti-CCR7–FITC (150503; R&D Systems, Abingdon, U.K.) and anti-CD45RO–PerCP-Cy5.5 (UCH1; BioLegend, London, U.K.). Naive T cells (Tns) were defined as CD45RO–CCR7+; central memory T cells (Tcm) were defined as CD45RO+CCR7+; effectector memory T cells (Tem) were defined as CD45RO+CCR7+; and terminally differentiated T cells (Temras) were defined as CD45RO+CCR7+ (28).

Surface expression of coinhibitory receptors was determined by staining cells with anti-CD279(PD1)–PECy7 (J105; eBioscience), anti-CD223 (LAG3)–PE (polyclonal; R&D Systems), anti-CD161–PE (688327; R&D Systems), and anti-CD244–allophycocyanin (eBioDM244; eBioscience). Flow cytometry was performed using a FACSCanto II (BD Biosciences). Gates for PD1 expression were set using an isotype-matched control Ab, and gates for LAG3, CD161, and CD244 expression were set on distinct positive populations. FACSDiva software (BD Biosciences) was used for analysis.

Allogeneic T cell stimulation using PBMCs

To quantify alloreactive T cell responses, PBMCs were labeled with 0.5 μM CFSE (Invitrogen, Paisley, U.K.), and 1*10⁶ recipient PBMCs were stimulated with 2*10⁴ irradiated (30 Gy) donor-derived or third-party–derived CD40-activated B cells. Third-party-derived CD40-activated B cells were expanded from splenocytes of an individual having the same number of HLA mismatches with the patient as the number of mismatches between patient and donor, but completely mismatched with the donor on HLA-A, HLA-B, and HLA-DR (27). Cocultures were performed in 96-well U-bottom plates in a final volume of 200 μl B cell medium (IMDM + 10% human serum + 1% Penicillin-Streptomycin [Life Technologies] + 1% Insulin-Transferrin-Selenium [Life Technologies]) (27). In addition, to determine responses to polyclonal stimulation, PBMCs were stimulated with PHA (5 μg/ml; Murex, Paris, France). Each assay was performed in duplicate. Flow cytometric analysis was performed after 5 d of culture at 37°C and 5% CO2. Cells were washed with PBS (Lonza), and staining for cell viability was performed using a LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen), according to the manufacturer’s protocol. Cells were then stained with anti-CD3–PerCP-Cy5.5 (UCHT1; BD Biosciences), anti-CD4–APC–H7 (SK3; BD Biosciences), anti-CD8–eFluor 450 (RPA-T8; eBioscience) to distinguish T cells, as well as anti-CD19–Horizon V500 (HIB19; BD Biosciences) to exclude B cells. Cytotoxic degranulation was detected using CD107a–allophycocyanin (eBioscience), which was added during the last 15 h of the cocultures. Cells were analyzed for proliferation using CFSE-dilution patterns and for phenotype on a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA). We used FACS Diva software (BD Biosciences) for analysis of phenotypic markers. Precursor frequencies (PFs), which are the proportions of the cells that respond to the stimulus, of alloreactive CD4+ and CD8+ T cells were calculated using ModFit LT software (Verity Software House), as previously described (27). Average PFs were calculated from duplicate assays.

Allogeneic T cell stimulation of sorted T cells

To compare the proliferative capacities of CD8+CD244+ and CD8+CD244– T cells, post-LTx PBMCs from patients of the late cohort were thawed and labeled with 0.5 μM CFSE. CFSE-labeled PBMCs were stained with anti-CD3–PerCP-Cy5.5 (UCHT1; BD Biosciences), anti-CD4–allophycocyanin–H7 (SK3; BD Biosciences), anti-CD8–eFluor 450 (RPA-T8; eBioscience), and anti-CD244–allophycocyanin (eBioDM244; eBioscience). CD8+CD244+, CD8+CD244–, and CD4+ T cells were purified by flow cytometric sorting, using a FACSaria Cell Sorter (BD Biosciences). Only cells with purity > 95% were used. Purified CD8+CD244+ or CD8+CD244– T cells (2*10⁴), together with purified autologous CD4+ T cells (2*10⁴), were stimulated with 1*10⁷ irradiated (30 Gy) donor CD40-activated B cells or third-party CD40-activated B cells, as described above. To study the role of coinhibitory receptor–ligand interactions in allogeneic T cell responses, ligands of coinhibitory receptors were blocked by the addition of neutralizing anti-CD270 (HVEM/TNFRSF14) (clone 94801; R&D Systems) (29) or anti-CD48 (eBio156-4H9; eBioscience) (10, 11) Abs, either alone or in combination, to selected allogeneic T cell stimulations. After 5 d of culturing, cells were stained, and ModFit analyses were performed as described above.

Determination of CMV-specific T cells

To determine the frequencies of CMV-specific CD8+ T cells in PBMCs and to assess whether these cells expressed CD244, 1*10⁶ PBMCs were stained with a mixture of HLA-A*01:01, HLA-A*02:01, HLA-A*11:01, HLA-A*24:02, HLA-B*07:02, HLA-B*08:01, and HLA-B*35:01 MHC class I tetramers loaded with Pp-derived, Pp65-derived, and IE-derived peptides (Department of Hematology, Leiden University Medical Center), depending on the HLA types of the patient. The following peptides were used: Pp: VTEHDTLLY (HLA-A*01:01); Pp65: YSEHPTFTSQY (HLA-A*01:01), NLYPMVATV (HLA-A*02:01), ATVGQNLK (HLA-A*11:01), KYAQKIFKIL (HLA-A*24:02), RPHRERNGFTVL (HLA-B*07:02), PTRVYGGAM (HLA-B*07:02), and PSIPNYVHY (HLA-B*35:01); and IE1: QIKVRDVMV (HLA-B*08:01) and ELRRKRMHYM (HLA-B*08:01). In addition, cells were stained with anti-CD4–PerCP (clone Leu3A SK7; BD Biosciences), anti-CD8–Pacific Blue (clone RPA-T8; BD Biosciences), and anti-CD244–allophycocyanin

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considered statistically significant.  

1840 CMV-INDUCED CD244 LEADS TO T CELL HYPORESPONSIVENESS 

CD8+ T cells was sustained late after LTx. Therefore, subsequent 

At 6 mo post-LTx, the expression of PD1 on CD4+ T cells had 

were tested using the Mann–Whitney 

SPSS for Windows (version 21.0 software package). A 

increase at 1 mo post-LTx on both CD4+ ( 

controls (Fig. 1C), indicating that liver disease had no influence on 

To establish whether the changes observed in the first 6 mo after 

By 1 mo after LTx, a slight increase in the expression of PD1 on 

returned to baseline levels, whereas CD244 expression on CD8+ 

as well as on all CD8+ T cell subsets, we found increasing expression of CD244 during the 

Upregulation of CD244 on CD8+ T cells after LTx is 

Next, we focused on the expression of CD244 and CD160 in 

Collectively, the slight increase in PD1 expression on CD4+ 

CD160, and CD244 (5), on circulating CD4+ and CD8+ 

Patient characteristics are depicted in Table I and described in Materials and Methods. Because TIM3 was barely expressed on T cells from 

not shown). Representative FACS plots are shown in Fig. 1A. 

By 1 mo after LTx, a slight increase in the expression of PD1 on 

and CD244 on CD8+ T cells was observed (Fig. 1B). At 6 mo post-LTx, the expression of PD1 on CD4+ T cells had returned to baseline levels, whereas CD244 expression on CD8+ T cells had increased further. In addition, expression of CD244 on 

upregulation of CD160 and CD244 expression on circulating T cells after LTx was caused by a shift in T cell subset distribution, as well as by the increased expression on the individual T cell subsets. 

Rapid and sustained increase in CD244 and CD160 expression 

Elastic graphic transformation was performed using an LSR II 

Flow cytometry was performed using an LSR II 

and data were analyzed using FACS Diva software. 

Statistical analysis 

All data are presented as mean ± SEM. All datasets were tested for normal 

Gaussian distribution using the Shapiro–Wilk normality test. The signifi- 
cance of differences between paired observations was tested using the 
paired t test for normally distributed data or the Wilcoxon signed-rank test for nonnormally distributed data. Differences between unrelated groups 

were tested using the Mann–Whitney U test. Statistical analyses were performed using GraphPad Prism (Version 5.01; GraphPad Software, San 

Diego, CA). Multivariate analysis was performed using linear regression in 

results. Representative FACS plots are shown in Fig. 1A. 

To establish whether the changes observed in the first 6 mo after 

LTX were sustained, we assessed coinhibitory receptor expression on 

circulating T cells in blood samples collected from 38 patients 

1–12 y after LTx and compared it with expression before LTx. 

In the early post-LTx cohort, we observed a significant increase 

in CD160 expression on CD8+ Tems and Tems 6 mo post-LTx 

(Fig. 2B). In the late post-LTX cohort, CD160 expression increased significantly after LTx on CD4+ Tems, Tems, and Temras (Fig. 2C). 

On CD4+ Tems and Temras, as well as on all CD8+ T cell subsets, we found increasing expression of CD244 during the first 6 mo after LTx in the early post-LTX cohort (Fig. 2B). In the late post-LTX cohort, CD244 expression on CD4+ and CD8+ Tems and Temras increased significantly after LTx (Fig. 2C). 

Taken together, these results show that the observed upregulation of CD160 and CD244 expression on circulating T cells after LTx was caused by a shift in T cell subset distribution, as well as by the increased expression on the individual T cell subsets. 

Uptregulation of CD244 on CD8+ T cells after LTx is 

associated with CMV infection 

Next, we focused on the expression of CD244 and CD160 in patients late after LTx. Because expression of CD244 and CD160 on 

T cells increases with age (31), and their expression can also be induced by viral infections, such as hepatitis B virus (HBV), HCV, 

and CMV (5, 10, 14, 32–34), we first asked whether the increasing expression of these co-inhibitory receptors after LTX was related to age of the patients, underlying disease (including chronic viral hepatitis), time after LTX, or CMV infection after LTX. Importantly, for this study, CMV infection was only regarded as relevant when occurring between LTX and collection of the post-LTX blood sample. Multivariate linear regression analysis showed that the increases in CD160 and CD244 expression (Δ expression = expression post-LTX − expression pre-LTX) were not significantly associated with patient age, time after LTX, or underlying disease. However, upregulation of CD244, but not CD160, on CD8+ T cells after LTX showed a significant positive association with CMV infection after LTX (p = 0.004) (Table II). Although we focused on the expression of CD244 and CD160, we also analyzed the association between CMV infection and the expression of PD1 and LAG3; however, no increase in their expression level was found in CMV-infected patients. 

Fig. 3A shows that the expression of CD244 on CD8+ T cells was significantly upregulated after LTX in patients with CMV infection but not in patients without CMV infection after LTX. The increases in CD244 expression observed in patients without CMV infection were small (on the average only 7%), whereas a signifi- 
cantly higher average increase of 38% was observed in patients with CMV infection (Fig. 3B). Therefore, we conclude that CMV infection is an important contributor to the increase in CD244 expression on CD8+ T cells after LTX. Together, our data suggest that strong CD244 expression is induced by CMV infection early after LTX and that CD244 expression remains high, even many years after CMV infection is cleared. To verify this, we determined CD244 expression levels on CD8+ T cells at 1 y post-LTX in a subgroup of CMV-infected patients (n = 6) from the long-term cohort. As shown in Fig. 3C, expression of CD244 was already increased in these patients at 1 y after LTX. Although expression levels showed a partial decrease in blood samples taken at 6–11 y post-LTX, they remained significantly higher than pre-LTX expression levels. These data indicate that CMV infection, which
occurs predominantly in the first 9 mo after transplantation, induces the accumulation of CD8+ T cells expressing CD244, and these CD8+CD244+ T cells persist after the clearance of infection (latency).

Reduced allogeneic CD8+ T cell responses in patients with CMV infection after LTx

Because we found that CMV infection after LTx was associated with upregulation of CD244 expression on CD8+ T cells after LTx, we asked whether CMV infection also affected the alloreactivity of these cells. Therefore, we determined CD8+ T cell alloresponses in patients from the late post-LTx cohort, with and without CMV infection, by coculturing CFSE-labeled patient PBMCs with CD40-activated B cells from their liver transplant donors or from an HLA-mismatched third party. After 5 d, proliferation and effector function of CD8+ T cells were assessed. PFs of proliferating cells were calculated using ModFit software (27); representative examples of ModFit proliferation analyses are shown in Fig. 4A. As depicted in Fig. 4B, PFs of CD8+ T cells proliferating in response to donor alloantigens were significantly lower in PBMCs from patients with CMV infection than from patients without CMV infection after LTx, with the same trend (p = 0.213) in the responses to third-party alloantigens.

To assess the cytotoxic effector function of CD8+ T cells in both patient categories, we determined their cytotoxic degranulation capacity by analyzing CD107a surface expression at the end of the cocultures (Fig. 4C). After stimulation with donor or third-party alloantigens, CD8+ T cells from patients with CMV infection after LTx showed significantly lower levels of CD107a expression than did CD8+ T cells from patients without CMV infection (Fig. 4D). More specifically, CD107a expression in nonproliferating cells was decreased in patients with CMV infection in contrast with CD107a expression in proliferating CD8+ T cells (Supplemental Fig. 2). These data demonstrate the existence of circulating CD8+ T cells that do not proliferate but are still capable of cytotoxic degranulation in response to alloantigens and show that the decreased allogeneic cytotoxic degranulation capacity in CMV-infected patients was confined to these nonproliferating cells.

Table I. Demographic and clinical characteristics of patients included in the early post-LTx and late post-LTx cohorts

<table>
<thead>
<tr>
<th>Early Post-LTx Cohort</th>
<th>Total: 19 Patients</th>
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</thead>
<tbody>
<tr>
<td>Recipient age (y; median [range])</td>
<td>43 (25–63)</td>
</tr>
<tr>
<td>Recipient gender, female (n [%])</td>
<td>10 (53)</td>
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<tr>
<td>Underlying disease (n [%])</td>
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<td>AHF</td>
<td>4 (21)</td>
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<td>HCC</td>
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<tr>
<td>Donor age (y; median [range])</td>
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<td>Donor gender, female (n [%])</td>
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<td>Basiliximab as induction immunosuppression (n [%])</td>
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<td>20 (53)</td>
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<td>18 (47)</td>
</tr>
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</table>

a One patient in the early post-LTx cohort received a combination of tacrolimus and everolimus.

b One patient in the early post-LTx cohort received a combination of tacrolimus and mycophenolate mofetil; four patients in the late post-LTx cohort received mycophenolate mofetil (two in combination with tacrolimus).

c Five patients in the late post-LTx cohort received everolimus (two in combination with tacrolimus).

d One patient in the late post-LTx cohort received a combination of rapamycin and tacrolimus.

AHF, acute hepatic failure; AIH, autoimmune hepatitis; HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; SBC secondary biliary cirrhosis.
Together, these data demonstrate a reduction in allogeneic CD8+ T cell–proliferative and cytotoxic-degranulation responses in LTx patients with CMV infection after LTx and show that CMV infection induces the accumulation of a population of dysfunctional CD8+ T cells that neither proliferates nor degranulates in response to allostimulation.
CD244+CD8+ T cells show impaired proliferative responses to allogeneic stimulation

Because we found that CMV infection was associated with a strong increase in CD244 expression on circulating CD8+ T cells and with hyporesponsiveness of CD8+ T cells to alloantigens after LTx, we wondered whether CD244 expression is a hallmark of a subpopulation of CD8+ T cells with reduced functionality. To test this hypothesis, CFSE-labeled CD244− and CD244+ CD8+ T cells, as well as CD4+ T cells, were sorted from 17 LTx patients from the late post-LTx cohort. The sorted CD8+ T cell subsets were cocultured with autologous CD4+ T cells to provide CD4 help to the CD8+ T cells and stimulated with allogeneic CD40-activated B cells derived from the donor or from an HLA-mismatched third party. In addition, both sorted subsets were stimulated with PHA. After 5 d of culture, cells were harvested, and proliferation was measured. Significantly lower numbers of CD244+ T cells compared with CD244− T cells proliferated in response to PHA (Fig. 5A, p = 0.0001). Similarly, significantly fewer CD244+ T cells than CD244− T cells proliferated in response to allogeneic stimulation (p = 0.002 for donor and p = 0.023 for third-party stimulation). The impaired proliferative responses of CD244+ T cells were independent of the alloantigenic source, because differences between CD244− and CD244+ T cells were similar in response to donor and third-party stimulations (Fig. 5B). To assess whether blocking the interaction of CD244 with its ligand CD48 could restore the proliferative capacity of CD244+CD8+ T cells, we repeated the above-described experiments in a subgroup of patients in the presence of blocking Abs directed against CD48. In addition, we studied the effect of blocking the interaction of CD160, which is coexpressed with CD244, with its ligand HVEM.

Table II. Associations of independent covariates with increasing (Δ) CD160 and CD244 expression on CD4+ and CD8+ T cells in multivariate linear regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔCD160 on CD4+ T Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age recipient</td>
<td>0.336</td>
<td>0.199</td>
</tr>
<tr>
<td>Time after LTx (y)</td>
<td>0.171</td>
<td>0.492</td>
</tr>
<tr>
<td>Underlying disease</td>
<td>0.047</td>
<td>0.842</td>
</tr>
<tr>
<td>CMV infection after LTx</td>
<td>0.357</td>
<td>0.165</td>
</tr>
<tr>
<td>ΔCD160 on CD8+ T Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age recipient</td>
<td>0.239</td>
<td>0.368</td>
</tr>
<tr>
<td>Time after LTx (y)</td>
<td>−0.287</td>
<td>0.269</td>
</tr>
<tr>
<td>Underlying disease</td>
<td>−0.048</td>
<td>0.843</td>
</tr>
<tr>
<td>CMV infection after LTx</td>
<td>0.434</td>
<td>0.106</td>
</tr>
<tr>
<td>ΔCD244 on CD4+ T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age recipient</td>
<td>0.469</td>
<td>0.069</td>
</tr>
<tr>
<td>Time after LTx (y)</td>
<td>0.180</td>
<td>0.450</td>
</tr>
<tr>
<td>Underlying disease</td>
<td>−0.066</td>
<td>0.769</td>
</tr>
<tr>
<td>CMV infection after LTx</td>
<td>0.232</td>
<td>0.337</td>
</tr>
<tr>
<td>ΔCD244 on CD8+ T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age recipient</td>
<td>−0.223</td>
<td>0.251</td>
</tr>
<tr>
<td>Time after LTx (y)</td>
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<td>0.388</td>
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<tr>
<td>Underlying disease</td>
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<td>0.410</td>
</tr>
<tr>
<td>CMV infection after LTx</td>
<td>0.631</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Δ expression, expression post-LTx − expression pre-LTx.
during culture. Both CD48 and HVEM were expressed on CD40-activated B cells (data not shown). After 5 d, we did not find any difference in allogeneic proliferation of CD244+ and CD244−CD8+ T cells between conditions with and without blocking Abs (data not shown). Collectively, these data suggest that CD244 expression marks a subset of dysfunctional CD8+ T cells, but the receptor itself and the coexpressed CD160 receptor do not mediate the dysfunctionality.

FIGURE 3. Expression of CD244 on CD8+ T cells before and after LTx in patients of the late post-LTx cohort with and without CMV infection after LTx. (A) Expression of CD244 on CD8+ T cells before and after LTx in patients from the late post-LTx cohort with and without CMV infection after LTx. (B) Increase in CD244 expression on CD8+ T cells (i.e., ΔCD244 = CD244 expression post-LTx − pre-LTx expression) in patients with and without CMV infection after LTx. (C) Expression of CD244 on CD8+ T cells before LTx (Pre-LTx) and at 1 and 6–11 y post-LTx in a subgroup of patients with CMV infection (n = 6) from the long-term cohort. Each symbol represents an individual patient, and lines indicate mean with SEM. *p < 0.05, **p < 0.005, ***p < 0.0005.

FIGURE 4. Allogeneic proliferative and cytotoxic degranulation responses of CD8+ T cells from patients with or without CMV infection after LTx. (A) Representative ModFit analysis plots showing CD8+ T cell proliferation after 5 d of stimulation with donor-derived or third-party–derived CD40-activated B cells. The blue peaks represent nondivided cells. Different generations of divided cells are depicted in different colors. (B) PFs of proliferating CD8+ T cells in post-LTx PBMCs from patients with or without CMV infection after LTx in response to donor (n = 14 with CMV, n = 9 without CMV) or third-party (n = 9 with CMV, n = 8 without CMV) alloantigens. Blood samples were collected 1–12 y after LTx (median, 7 y). (C) Representative FACS plots showing CD107a expression on CFSE-labeled CD8+ T cells after 5 d of stimulation with donor-derived CD40-activated B cells. (D) CD107a expression on proliferated and nonproliferated CD8+ T cells as the percentage of all CD8+ T cells in post-LTx PBMCs from patients with (n = 8) or without (n = 7) CMV infection after LTx in response to donor or third-party alloantigens. Each symbol represents an individual patient, and lines indicate mean with SEM. *p < 0.05.
CD244+CD8+ T cells contain the majority of CMV-specific cells

Because upregulation of CD244 on circulating CD8+ T cells was strongly associated with CMV infection after LTx, and CD8+ CD244+ T cells were dysfunctional, we analyzed whether CD244+ CD8+ T cells contained CMV-specific cells. We obtained PBMCs from five CMV-experienced LTx patients from our study with MHC class I tetramers loaded with CMV peptides and CD244 mAb, as described in Materials and Methods. A representative FACS plot is shown in Fig. 6A. We found that the CD244+CD8+ T cell population contained the majority of CMV tetramer+ cells, significantly more than the CD244-CD8+ T cell population (Fig. 6B, p = 0.033). These data support a causal relationship between CMV infection and the expansion of dysfunctional CD8+ CD244+ T cells after LTx.

Discussion

In this study, we showed that the coinhibitory receptor CD160 was upregulated on circulating memory CD8+ T cells, whereas the coinhibitory receptor CD244 was upregulated on both CD4+ and CD8+ memory T cells of patients early after LTx. In addition, we found that the increased expression of both receptors was sustained late after LTx. In contrast, PD1 was transiently upregulated on CD4+ T cells 1 mo after LTx, but its expression had already normalized at 6 mo after LTx. Our original hypothesis postulated that the long-term persistence of a high load of alloantigens after LTx may induce exhaustion of allogeneic T cells. In contrast to this hypothesis, impaired allogeneic CD8+ T cell responses and strong upregulation of CD244 on CD8+ T cells after LTx were restricted to patients with CMV infection after LTx. Expression of CD244 on the majority of CMV-specific CD8+ cells in patients with CMV infection after LTx suggested a causal relationship between CMV infection and the observed expansion of CD8+ CD244+ T cells. The observed lower proliferative capacity of CD244+CD8+ T cells compared with CD244- CD8+ T cells in response to allogeneic stimulation suggested that the allogeneic CD8+ T cell hyporesponsiveness in LTx patients after CMV infection is caused by the accumulated CD8+CD244+ T cells. Together, these data suggest that CMV infection after LTx induces persistent accumulation of CD8+CD244+ T cells in the circulation, which display features of senescence or exhaustion, resulting in impaired peripheral CD8+ T cell responses to alloantigens in these patients.

The observed association between the accumulation of CD8+ CD244+ T cells and CMV infection (11, 14, 30, 35), as well as the selective expression of CD160 and CD244 on memory T cell subsets, is consistent with previous studies (4, 11, 30, 36). However, to our knowledge, this is the first study showing that CMV infection after organ transplantation induces sustained CD244 expression on memory CD8+ T cells and that the resulting CD8+ CD244+ T cell subset is hyporesponsive to alloantigens. It is well documented that CMV infection induces vast expansion in the circulation of a population of CMV-specific CD8+ T cells that are actively cycling. After establishment of CMV latency, the majority of CMV-specific CD8+ T cells become long-lived terminally differentiated resting T cells with poor proliferative capacity (37, 38). Therefore, we hypothesize that expansion of these cells after LTx occurs during active CMV infection, while they differentiate into long-lived CD244-expressing terminally differentiated T cells with poor proliferative capacity after establishment of CMV latency. Expression of CD244 is also induced on CD8+ T cells by HIV and HCV infection and results in impaired CD8+ T cell responses to viral Ags. However, the impairment of antiviral CD8+ T cell responses in these patients can be abrogated by blocking the interaction between CD244 and its ligand CD48 (10, 11, 14). In contrast, our data suggest that the observed allogeneic hyporesponsiveness of CMV-induced CD244+CD8+ T cells was not mediated by CD244-CD48 interaction or by interaction of the coexpressed inhibitory CD160 receptor with its ligand HVEM, because blocking CD48 or HVEM did not lead to abrogation of hyporesponsiveness of CD8+CD244+ T cells to alloantigens in experiments with sorted CD244+ T cells. The impaired response of this subset to alloantigens may be related to its high content of CMV-specific T cells, resulting in lower proportions of T cells with other specificities, including alloreactive T cells (39–41). In addition, the limited TCR repertoire of CMV-specific cells (39, 42, 43) accumulated in this subset may result in poor cross-reactivity to directly presented alloantigens. However, these phenomena do not explain the impaired proliferation of the expanded CD8+ CD244+ T cells to PHA (this study) or to CD3/CD28 stimulation in a previous study (30). Interestingly, CMV-induced expansion of CD8+ effector memory cells correlates with a decrease in T cell telomere length, indicating T cell senescence (44), and T cell

A Proliferation in response to PHA

B Proliferation in response to donor and third party

FIGURE 5. Proliferation of CD244+ versus CD244-CD8+ T cells in response to polyclonal and allogeneic stimulation. (A) PFs of sorted CD244+ versus CD244-CD8+ T cells in response to polyclonal stimulation (PHA). (B) PFs of CD244+ versus CD244-CD8+ T cells of patients post-LTx in response to donor and third-party stimulation. Cells were sorted from PBMCs collected from 17 patients 2–10 y after LTx (median, 6.2 y). Each symbol represents an individual patient, and lines indicate mean with SEM. *p < 0.05, **p < 0.005, ***p < 0.0005.
senescence has been related to impaired proliferative capacity (45). Therefore, we propose that the observed increase in CD244 expression in LTx patients with CMV infection marks expansion of a subset of highly differentiated, but dysfunctional, CD8+ T cells that shows features of senescence or exhaustion. However, its proliferative capacity is hampered by an unknown mechanism.

The observed reduction in alloreactive CD8+ T cell responses in LTx patients after CMV infection challenges the broadly accepted notion, based on experimental animal studies, that viral infections stimulate heterologous immunity, resulting in increased frequencies of alloreactive T cells (46). Indeed, reactivation of CMV infection, as well as primary CMV infection, abrogates transplant acceptance in mice and rats (47, 48). However, several previously published observations in humans support our findings. First, CMV infection leads to T cell senescence and, thereby, impairs T cell responses to other Ags and to vaccinations (34, 40, 49–51). Interestingly, CMV infection after LTx is associated with an increased predisposition to develop opportunistic infections (52). Second, immune senescence has been associated with improved kidney allograft survival (53). Third, the majority of CD8+CD244+ T cells in our patients belong to the Temra subset, and accumulation of circulating CD8+ Temras recently was shown to be associated with a lower risk for acute rejection after kidney transplantation (41). It was not feasible to investigate whether CMV infection or accumulation of CD8+CD244+ cells was associated with differences in clinical outcome, such as graft or patient survival or acute rejection, in our long-term study cohort, because all patients have stable graft function and are still alive. In addition, only five patients suffered from acute rejection. A larger prospective study is required to investigate associations between CMV infection or an increase in CD244 and clinical outcomes.

The mechanism by which CMV infection induces expansion of CD244+CD8+ memory T cells is unknown, but it may be related to bystander effects of inflammatory responses caused by CMV or its immune-evasion strategies. A recent mouse study showed that CD244 is more highly upregulated during secondary, than during primary, CD8+ T cell responses, suggesting that T cell reactivation is required for the induction of high CD244 expression (54). In addition, it was shown that chronic lymphocytic choriomeningitis virus or Toxoplasma infections in mice impair memory T cell responses against unrelated Ags as a result of the generation of CD8+ Temras. This was caused by increased IFN-type signaling due to chronic inflammation caused by the persistent infections (55). A similar mechanism may be driven by CMV infection after LTx, which also causes inflammation in the graft and in other organs (52), whereas CMV is able to induce IFN-α production (56). A possible relationship between inflammation and reduced alloresponses after LTx is supported by a recent study that showed that chronic HCV patients who are operationally tolerant after LTx overexpress type I IFN and IFN-stimulated genes in the liver graft (57). A second possible explanation for the association between CMV infection and CD8+ T cell hyporesponsiveness is that CMV produces viral IL-10 (58), which inhibits the expansion of alloreactive CD8+ T cells. A third explanation may be that the immunological space of the recipient is occupied by large quantities of CMV-specific CD8+ Temras that compete with and, thus, hamper the expansion of T cells with other specificities (59). However, these explanations remain speculative, and further research is needed to decipher the mechanisms by which CMV infection induces expansion of CD8+CD244+ memory T cells, which is beyond the scope of the current study.

In contrast to the sustained increase in CD244 and CD160 expression on circulating T cells after LTx, we found that PD1 was only transiently upregulated following LTx. Because PD1 can be upregulated by TCR activation, this finding may be explained by the early and transient activation of donor-specific T cells after LTx that we observed previously (27). However, the use of calcineurin inhibitors by the majority of our patients may prevent sustained upregulation of PD1, because PD1 induction by TCR ligation involves NFAT signaling, which is inhibited by calcineurin inhibitors (32). PD1 upregulation is also prevented by mTOR inhibitors (60), immunosuppressive drugs used by a small group of patients in our cohorts. LAG3 and TIM3 expression did not show an increase after LTx, but we do not know whether this is related to the use of immunosuppressive drugs because no data exist on the effect of immunosuppressive drugs on the expression of these receptors. A limitation of our study is that we were not able to link the CD8+ T cell hyporesponsiveness to an immunologically tolerant state toward the liver allograft. To investigate the clinical impact of the findings presented in our study, it will be interesting to determine the effect of CMV infection in LTx patients on the success rate of withdrawal of immunosuppressive drugs. A prospective study in which immunosuppressive drugs are weaned is needed to investigate this.

In conclusion, we showed that CMV infection after LTx was associated with the expansion of CD8+CD244+ T cells with im-

FIGURE 6. CMV-specific cells within CD244+ and CD244−CD8+ T cell populations. (A) Representative FACS plots showing CD244+ and CD244− CMV tetramer+ CD8+ T cells. (B) Percentage of CD244+ and CD244− CMV tetramer+ cells of CD8+ T cells from CMV+ patients post-LTx (n = 5).
paired proliferative capacity in response to alloantigen, causing allodigenic CD8+ T cell hyporesponsiveness. These results suggest that CMV infection may hamper T cell immunity and, thereby, promote immunological graft acceptance after LTx.

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Disclosures
The authors have no financial conflicts of interest.

References


