CCL8 and the Immune Control of Cytomegalovirus in Organ Transplant Recipients

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Introduction

Monitoring virus-specific, cell-mediated immunereresponses is a promising way to predict CMV reactivation, and may ultimately be useful in the prevention and management of CMV infection among transplant recipients (1). In particular, CMV-specific cytotoxic T cells seem to be an important biomarker for predicting virologic and clinical outcomes. For example, assessments of CMV-specific IFN-γ responses pretransplant (2) or upon completion of antiviral prophylaxis (3,4) have been shown to predict CMV disease. Recently, we showed that the CMV-specific IFN-γ response measured at the onset of CMV viremia in SOT recipients had the potential to predict spontaneous clearance versus progressive viral replication (5). Cell-mediated immunity tests can not only help define risk groups for CMV disease, but also determine subgroups of patients capable of achieving suppression of viral replication exclusively by immunological means—an elusive goal at the present stage of CMV vaccine development (6). However, cytotoxicity exerted by CMV-specific T cells is only one component of the highly complex multipronged cellular and humoral response leading to immune control of the virus. An understanding of other factors and players involved in immunologic control of CMV beyond the accepted roles of cytotoxic T cells may facilitate the development of novel and improved preventative strategies.

The objective of this study was to perform an in-depth analysis of CMV cell-mediated immune responses by comprehensively profiling cytokine and chemokine expression upon ex vivo stimulation of whole blood from transplant recipients shortly after the onset of CMV viremia. We hypothesized that immune control of viral replication would be associated with distinct cytokine and chemokine responses, which were expected to offer new insights about the contributions of other peripheral blood cell subpopulations to the immunologic control of infection. As our findings supported the prominent role of two...
chemokines in the immune control of CMV viremia, we then elected to investigate further one of them, CCL8, as to its cellular origin and regulation by immunosuppressive agents. Among a second cohort of transplant recipients, we explored the use of genetic variability in this chemokine promoter for prediction of CMV viremia.

Materials and Methods

Patients, samples, and outcomes

Two distinct solid organ transplant recipient cohorts were analyzed using samples obtained from two previously published studies (4,5). For the first group (n = 37) (5), blood samples collected at or near the onset of asymptomatic CMV viremia were utilized for cytokine and chemokine profiling. Patients were followed clinically and with weekly CMV PCR. The outcomes assessed in this cohort were either spontaneous viral clearance or progressive viremia/symptomatic CMV disease requiring intravenous ganciclovir or oral valganciclovir. The second group (n = 67) comprised of CMV donor seropositive/receiver seronegative ID+ (R-) transplant recipients who were previously enrolled in noninterventional clinical trials at two Canadian centers (4). All patients received 3-6 months of antiviral prophylaxis, and blood samples collected upon discontinuation of treatment were utilized for single nucleotide polymorphism genotyping. The outcomes assessed in this cohort were the development of CMV viremia and CMV disease up to 1 year posttransplant. Definitions of CMV viremia and CMV disease were derived from the respective original studies. In addition, self-declared healthy adult volunteers (n = 8) donated blood samples, which were used to investigate the blood leukocyte origins of cytokines and chemokines. The original studies and the required amendments were reviewed and approved by the local Ethics Research Board. All patients and healthy volunteers provided written informed consent.

Plasma cytokine and chemokine measurements

In the first cohort (n = 37 individual patient samples at onset of CMV viremia), whole blood was incubated overnight in the presence or absence of 22 CMV peptides utilizing the commercially available QuantiTEN-CMV assay (QTF; Qiagen, Valencia, CA) according to the manufacturer’s instructions. Quantitation of 65 cytokines and chemokines (EGF, Eotaxin/CCL11, FGF-2, Flt-3 ligand, Fractalkine/CX3CL1, G-CSF/CSF3, GM-CSF/CSF2, GRO/CXCL1, IFN-α, IFN-γ, IL-10, IL-12(p70)/IL12B, IL-12(p70)/IL12A, IL-13, IL-15, IL-17/IL17A, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10/CXCL10, MCP-1/ CCL2, MCP-3/CCL3, MDC/CCL22, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-AA, PDGF-AB/BB, RANTES/CCL5, TGF-α, TNF-α, TNFβ, VEGF, sCD40L, sIL-2Rα, MCP-2/CCL8, MCP-4/CCL13, ENA-78/CXCL5, SDF-1α + β/CXCL12, BCA-1/ CXCL13, I-309/CCL1, MIP-1β/MIP-5/CCL15, TARC/CCL17, E-Cadherin/CCL21, EOTAXIN-2/CCL24, EOTAXIN-3/CCL26, CTACK/CCL27, IL-23, IFNα, IFNβ, TRAIL/TNFsf10, SCF/KITLG, TSLP, IL-20, IL-21, IL-28, IL-16, IL-33/IFN-HEV) was performed on the resultant stimulated and unstimulated plasma using Milliplex Map Human Cytokine/Chemokine kits (Millipore, Billerica, MA) according to the manufacturer’s protocol. The assays were performed using the LuminexTM 100 system (Luminex, Austin, TX) by Eve Technologies Corp. (Calgary, AB, Canada). Results beyond the extremes of the standard curves were attributed to either the lowest or the highest standard concentration of the respective analyte.

Cytokine/chemokine-based classification of viremia outcomes

In order to reduce the complexity of the data to the optimal number of cytokines and chemokines capable of accurately classifying patients into a particular outcome (spontaneous viral clearance vs. progressive viremial), the Gene Expression Model Selector (GEMS) (7) system was used. The software defines the optimum algorithms and parameters to support vector machine-based classification through comparative performance assessment generated by nested cross-validation procedures. A standard SVM with Gaussian kernel was employed for classification, utilizing z-normalized cytokine/chemokine ratios (vs. unstimulated controls); the optimal method devised for the data was a signal-to-noise ratio in a one-versus-rest fashion.

Single nucleotide polymorphism (SNP) genotyping

Patients in cohort 2 (n = 67 D + R- patients followed for CMV infection after antiviral prophylaxis) were genotyped for a CCL8 SNP. A custom TaqMan SNP qPCR assay for SNP rs3138035 was designed by Life Technologies (assay ID AHA9YJ1) based on the CCL8 promoter reference sequence obtained from NCBI. Synthetic oligonucleotides (Integrated DNA Technolo- gies, Coralville, IA) were utilized to validate the qPCR assay. Plasma genomic DNA automated extraction was performed using the DNA Investigator Kit (Qiagen). The qPCR was performed using a StepOne Real-Time PCR instrument and results were analyzed using the StepOne Software v2.2 (Life Technologies, Carlsbad, CA).

Cellular origin of CCL8 and IFN-γ

Peripheral blood mononuclear cells (PBMCs) were obtained from CMV-seropositive (n = 4) and CMV-seronegative (n = 4) samples by Ficol gradient centrifugation of whole blood and cryopreserved until further use. After thawing, cells were washed and resuspended in RPMI 5% human serum at a density of 5 × 10^6 cells/mL and stimulated overnight at 37 °C in QuantFeron NIL or CMV antigen tubes. Cell viability was confirmed using amine-reactive dye (LIVE/DEAD stain, Life Technologies). Fc receptors were blocked (eBioscience, San Diego, CA) prior to surface staining with antibodies (eBioscience, Biologend [San Diego, CA], or BD Biosciences [Franklin Lakes, NJ]) belonging to panel 1 (CD3-PerCP-Cy5.5, CD4-PE-CY7, CD6-APC-H7 and CD19-APC) or panel 2 (CD3-PerCP-Cy5.5, CD14-PE, CD19-APC and CD6- APCCy7). Antigen choices were based on the standardized immunopheno- typing proposed for the Human Immunology Project (8). Sorted leukocyte populations included monocytes (CD3−/CD14+/CD16−), NK cells (CD3+/ CD19−/CD14−/CD56+), B cells (CD3−/CD19+), CD4+ T cells (CD3+/ CD8−/CD4+), and CD8+ T cells (CD3+/CD8+/CD4−). Cells were maintained on ice throughout the staining process and formaldehyde fixation was not performed in order to better preserve their RNA content. Cell sorting and purity checks were performed in FACSAnA III (BD Biosciences). Cells were sorted directly into lysis buffer and RNA was extracted using an RNasey Plus micro kit (Qiagen). Reverse transcription was performed using High Capacity cDNA Reverse Transcription kits followed by qPCR using TaqMan assays (CCL8- hs01877151_m1, IFN-γ- hsa0539291_m1; RPL13A-Hs04194366_m1 and NRAS- Hs00906995_g1; allLife Technologies). Each PCR reaction contained the cDNA equivalent of 10 ng of total RNA, as quantified by spectrometry (NanoDrop, Thermo Scientific, Waltham, MA). Relative quantitation against unstimulated cells (i.e., NIL tube) was performed by the delta-delta-Ct method using RPL13a (9) as the endogenous control for B, T and NK cells; and 18S rRNA for monocytes. These were selected based on the smallest level of variation found between CMV and NIL samples of CMV seropositive healthy volunteers (data not shown).

Effect of immunosuppression on monocyte CCL8 expression

THP-1 cells cultured in RPMI 10% FCS were plated at a density of 200 000 cells/well in 96-well plates and pretreated for 4 h at 37 °C with serial dilutions of the immune suppressive agents tacrolimus, mycophenolate mofetil and rapamycin (all Enzo Life Sciences, Farmingdale, NY). The dose range for each drug was defined to ensure comparable metabolic activity between treated and untreated cells, as per MTT assay (data not shown; Roche, Indianapolis, IN). IFN-γ was added (final concentration 1 ng/mL; Peprotech, Rocky Hill, NJ) and cells were incubated for an additional 6 h.
before RNA extraction. Cells were lysed in plate and automated RNA extraction was performed using an RNeasy Micro kit (Qiagen).

**Statistical analysis**
Two-tailed testing was applied to all analyses, with \( p < 0.05 \) considered to be statistically significant. To facilitate comparison with previously published data, during the receiver operating characteristic curve analysis we subtracted background values found in the unstimulated blood (i.e. NIL tube) from the values measured in the CMV peptide-stimulated blood, analogous to the QuantiFERON-CMV manufacturer recommendations for IFN-\( \gamma \). All analyses were performed using PASW Statistics 18.0.0 (IBM, Armonk, NY) or Prism 6 (Graphpad, La Jolla, CA).

**Results**

**CMV peptide stimulation broadly alters the blood expression of cytokines and chemokines**
To analyze the blood secretome in the context of an MHC class I-restricted CMV peptide-triggered interferon-\( \gamma \) response, 65 cytokines and chemokines were measured in paired nonstimulated and CMV peptide-stimulated plasma samples of 37 organ transplant recipients at the onset of CMV viremia. Quantitation within the assay limits was achieved in the majority of the specimens for 60 of the 65 cytokines/chemokines (Table S1). Of the five remaining cytokines/chemokines, IL-3, IL-9, IL-21, IL-28A, and CCL26 were detected in less than 50% of paired patient samples. Table S2 summarizes the effects of peptide stimulation on the levels of cytokines and chemokines. CMV peptides triggered significant up-regulation in expression of several proteins beyond IFN-\( \gamma \), in addition to promoting down-regulation of a selected set of cytokines and chemokines.

**Responses to CMV peptide stimulation differ according to viremia outcomes**
For this cohort of CMV-viremic transplant recipients, those who had spontaneous clearance of viremia showed a significantly higher ex vivo production of IFN-\( \gamma \) compared to those who had progressive viremia (5). We next compared the levels of cytokines/chemokines between patients with these two clinical outcomes. First, we analyzed background levels of cytokines and chemokines present in the nonstimulated blood samples. Univariate analysis revealed that only minimal background cytokine/chemokine differences between the two groups in the nonstimulated samples (data not shown). We then performed a univariate analysis to determine the cytokine/chemokine expression levels following CMV antigen stimulation, relative to their background levels, in patients with spontaneous clearance versus viral progression (Table 1; Tables S3 and S4). Patients achieving spontaneous clearance of viremia showed a distinct secretome with increased expression of 15 and decreased expression of nine cytokines/chemokines upon CMV peptide stimulation.

### Cytomegalovirus Immune Containment and CCL8

#### Generating a cytokine/chemokine-based classifier of viremia outcomes
We postulated that identifying the specific cytokine and chemokine contributions that lead to the most precise classification of virologic outcomes during monitored CMV replication episodes would improve our understanding of the hierarchy of inflammatory mediators involved in the immunologic control of replication. We utilized the Gene Expression Model Selector (GEMS) (7) to devise highly accurate classification models based on cytokine/chemokine level changes promoted by CMV peptide stimulation (i.e. CMV:NIL ratio). The measured effects of CMV peptide stimulation yielded a classifier with an accuracy of 80% in a 10-fold cross-validation performed on the same dataset. The cytokines and chemokines that most contributed to the classification model are ranked in Table 2. CCL8, IFN-\( \gamma \), and CXCL10 demonstrated the highest increase in expression upon CMV peptide stimulation and were closely correlated (CCL8 and CXCL10: Spearman’s rho 0.642, \( p < 0.001 \); IFN-\( \gamma \) and CCL8: Spearman’s rho 0.912, \( p < 0.001 \); IFN-\( \gamma \) and CXCL10: Spearman’s rho 0.571, \( p < 0.001 \)).

#### CCL8 and CXCL10 as isolated predictors of viremia outcomes
The discriminatory power of CMV peptide-elicited IFN-\( \gamma \) production to predict spontaneous clearance of viremia has been defined previously for this sample set (AUC 0.843 95% CI 0.709–0.977; \( p = 0.003 \)) (5). For comparison purposes, the levels of other cytokines and chemokines following CMV peptide stimulation of whole blood were also individually analyzed. Receiver operating characteristic curves confirmed the discrimination capacity of CCL8 (AUC 0.849, 95% CI 0.721–0.978; \( p = 0.003 \)) and CXCL10 (AUC 0.841, 95% CI 0.707–0.974; \( p = 0.004 \)). No other cytokine/chemokine demonstrated a statistically significant discriminatory power following CMV peptide stimulation (Figure 1).

#### Monocytes are the source of CCL8
To better contextualize cytokine and chemokine production in the antiviral response evoked by CMV peptide stimulation, we next sought to identify the cell types primarily responsible for producing the top-ranked cytokine (IFN-\( \gamma \)) and chemokine (CCL8) in the generated classifier. For that, PBMCs from QTF-CMV positive and negative healthy individuals were incubated overnight in QTF CMV or NIL tubes, and sorted by flow cytometry (Figures S1 and S2), yielding cell phenotypes of high purity (>95%) suitable for studying mRNA gene expression. IFN-\( \gamma \) mRNA transcription was evident in CD4+ and CD8+ T cells of CMV seropositive individuals. Higher IFN-\( \gamma \) levels were also detected in NK cells from these individuals (Figure 2). Amongst the leukocyte populations analyzed, monocytes were the only cell type in which CCL8 mRNA could be detected, with CMV-seropositive individuals showing median
expression levels 2-log_{10} higher than CMV-seronegative individuals in the CMV-stimulated sample (Figure 2).

**CCL8 promoter polymorphism is associated with risk of CMV viremia following donor-derived primary CMV infection**

A potentially relevant factor affecting cytokine/chemokine levels is the genetic variability among hosts. In particular, polymorphisms in the gene promoter region may impact the epigenetic regulation of gene expression. Using a TaqMan-based SNP assay, we determined the CCL8 promoter SNP rs3138035 genotypes of 67 D+/R− transplant recipients. Individuals who were homozygous for the CCL8 promoter minor allele (TT) were significantly more likely to experience viremia after discontinuation of primary antiviral prophylaxis (Log-rank Mantel–Cox test for difference between CC/CT/TT curves p = 0.0178; Figure 3). These individuals had a risk of viremia that was 3.62 times greater than carriers of at least one major C allele (logrank hazard ratio; 95% CI 2.077–51.88). There was no association between CCL8 promoter genotypes and the development of symptomatic CMV disease in the follow up (Log-rank Mantel–Cox test p = 0.7114).

**Immunosuppression adversely affects the production of CCL8**

Immunosuppressive drugs are known modulators of cytokine/chemokine expression in response to CMV antigens (10). We hypothesized that the expression of CCL8 is also affected by therapeutic immunosuppression. Known inducers of CCL8 expression include IFN-γ, IL-1β (11,12), and TNF-α (13). Since the expression of both IL-1β and TNF-α was reduced by CMV peptide stimulation (Table S3) independent of viremia outcomes, we assessed the effects of immunosuppression on IFN-γ-induced CCL8 expression in monocytes. Undifferentiated monocytic THP-1 cells pretreated with and subjected to increasing concentrations of rapamycin, tacrolimus or mycophenolate were stimulated with a fixed IFN-γ dose. We observed a dose-dependent inhibitory effect on CCL8 mRNA expression (Figure 4) that was especially pronounced for tacrolimus, implicating these drugs in the regulation of monocyte chemokine responses.
Discussion

The immune response against CMV is both complex and diverse. In the current study, we further characterize it by comprehensively profiling the expression of cytokines and chemokines following ex vivo CMV peptide stimulation. Beyond the established importance of T cell responses as a biomarker for CMV outcomes in transplantation, we evaluate the relevance of other host immune cells and chemokines in the efficient immunologic control of CMV. In particular, we demonstrate that the monocytic chemokine CCL8 produced in response to ex vivo CMV peptide stimulation is associated with spontaneous viral clearance in patients with CMV viremia. We additionally show that a mutation in the promoter region of CCL8 is associated with an increased risk of CMV replication in high-risk D+/R−/C0 transplant patients. Finally, we demonstrate that monocytes are the main source of CCL8 production in response to CMV stimulation and that CCL8 expression is modulated by commonly used immunosuppressive drugs. While important as a predictor of CMV outcomes, the prototypic cytotoxic CD8+ T cell response is only one part of a larger dynamic network of other relevant and interdependent immune cells, in which chemokines play an often underestimated role.

Clinically, in transplantation, chemokine expression has not only been associated with response to infections, including CMV, but also with allograft rejection, as has been demonstrated for CXCL10, for example (14–17).

Table 2: Relative importance of cytokines and chemokines when classifying patients according to CMV viremia outcomes

<table>
<thead>
<tr>
<th>Rank</th>
<th>Protein</th>
<th>Progressive viremia fold-change</th>
<th>Spontaneous Clearance fold-change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCL8</td>
<td>+1.20</td>
<td>+5.62</td>
</tr>
<tr>
<td>2</td>
<td>IFN-γ</td>
<td>−1.19</td>
<td>+1.91</td>
</tr>
<tr>
<td>3</td>
<td>CCL5</td>
<td>+1.12</td>
<td>+1.56</td>
</tr>
<tr>
<td>4</td>
<td>CXCL10</td>
<td>−1.01</td>
<td>+2.01</td>
</tr>
<tr>
<td>5</td>
<td>CCL4</td>
<td>−3.03</td>
<td>−1.54</td>
</tr>
<tr>
<td>6</td>
<td>CCL2</td>
<td>−1.14</td>
<td>+1.04</td>
</tr>
<tr>
<td>7</td>
<td>PDGF-AB/BB</td>
<td>−1.02</td>
<td>+1.12</td>
</tr>
<tr>
<td>8</td>
<td>IL-4</td>
<td>−1.04</td>
<td>+1.37</td>
</tr>
<tr>
<td>9</td>
<td>IL-8</td>
<td>−1.41</td>
<td>−2.08</td>
</tr>
<tr>
<td>10</td>
<td>CCL3</td>
<td>−5.00</td>
<td>−3.70</td>
</tr>
</tbody>
</table>

The 10 top-ranked cytokine and chemokine responses in whole blood after CMV MHC-I-restricted peptide stimulation contributing to an average classification accuracy of 80% are summarized, along with respective median fold-changes seen in patients with spontaneous viral clearance or progressive viremia requiring antiviral therapy. Positive and negative fold-changes correspond to increased or decreased levels upon CMV peptide stimulation, relative to background levels. Shading (p < 0.05) and underlining (p < 0.001) indicate statistically significant fold-changes based on a two-tailed paired Wilcoxon signed-rank test.

Figure 1: Performance of IFN-γ, CCL8, and CXCL10 in the prediction of immune clearance of CMV viremia. Protein levels of cytokines/chemokines elicited by whole blood stimulation with CMV MHC-I-restricted peptides were tested as predictors of spontaneous immune clearance of CMV viremia among 37 organ recipients. Receiver operating curve analyses were performed using protein concentrations measured in background-subtracted, peptide-stimulated samples. AUC: area-under-the-curve.
Mechanistically, aside from their roles in leukocyte trafficking between bone marrow, bloodstream, secondary lymphoid organs and tissues, chemokines are also key elements involved in T-helper cell polarization (18) and T cell differentiation (19). CCL2, a chemokine with high homology to CCL8, aside from its role in promoting the influx of leukocytes in infected tissues (20,21), is critical to both generation and survival of memory CD8 T cells (20). CCL8 elicits chemotaxis of activated T cells primarily through CCR5 (22), a receptor present in CMV-specific CD4+ T cell subsets (23), including central memory and effector/memory phenotypes (24). Among CD8+ T cells, CCR5 expression is highest in naïve cells (25), likely favoring cross-priming by monocytes (26), and is up-regulated in clonally proliferating antigen-experienced CD8+ T cells in response to their CMV cognate ligands (27). Monocyte expression of CCL8 in response to IFN-γ in a milieu comprised of other collaborating chemokines and cytokines would be aimed at priming naïve CD8 T cells and differentiating central memory into effector/memory CD8+ T cells, thereby promoting an integrated and efficient antiviral response. CMV, however, is very proficient at manipulating the host, and the multitude of virally-mediated immune evasion mechanisms targeting chemokines further corroborates their central role in achieving virologic control. For example, during lytic infection the viral IE-2 protein blocks the expression of chemokines CCL8, CCL5, CCL3, and IL-8 by CMV-infected fibroblasts (28). Conversely, CMV hijacks the host chemokine system of latently-infected CD34+ progenitor cells to attract CCR5+ CD4+ T cells via increased expression of CCL8, only to suppress their effector function with two other components of its secretome, TGF-β and IL-10 (29). The immune control of viral replication would therefore be contingent on overcoming the viral maneuvers that otherwise subvert the host chemokine system.

Another layer of regulation of CCL8 expression is determined by therapeutic immunosuppression, at both transcriptional and posttranscriptional levels. CCL8 expression depends on transcription factor phosphorylation by the mitogen-activated protein kinases ERK 1/2 and p38 (30), which occurs following IFN-γ stimulation in monocyctic THP-1 cells (31). This can be inhibited by tacrolimus (32) and mycophenolic acid (33). Posttranscriptional regulation of
IFN-\(\gamma\)-induced transcript stability is also dependent on such enzymes (34,35), with evidence indicating a faster decay of mRNA transcripts in association with calcineurin inhibitors (36) and rapamycin (37). In the present work, by using a human cell line we were able to study the in vitro impact of therapeutic immunosuppression on CCL8 while minimizing the influence of confounders potentially affecting this chemokine’s expression levels (e.g. genetic variability and CMV serostatus of the human cell donors). Within the limitations inherent to the cells chosen for the experiment, we observed a dose-dependent decrease in CCL8 gene expression in THP-1 cells pretreated with tacrolimus, mycophenolate mofetil or rapamycin, which is most likely consequent to the interaction between these two mechanisms, and indicates that the effects of therapeutic immune suppression go beyond their intended T and B cell targets, also affecting chemokine expression by monocytic antigen-presenting cells. This is particularly intriguing, given the clinically observed protective effect of inhibitors of the mammalian target of rapamycin (mTOR) against CMV infection (reviewed in 38). While down-regulating expression of chemokines both in THP-1 cells and in primary monocytes (39), under proinflammatory conditions rapamycin, modulates monocyte-derived dendritic cell cytokine production, leading to induction of IFN-\(\gamma\) expression in interacting T and NK cells (40), a prototypic response associated with the immune containment of viral replication.

In CMV cell-mediated immunity assays, IFN-\(\gamma\) is commonly utilized as a biomarker of a virus-specific, T cell-centered immune protective response. Interestingly in our study, two chemokines stood out for their equivalent performance as biomarkers for prediction of immune clearance of viral replication—CCL8 and CXCL10—with CCL8 in particular...
showing the largest fold-change. While of no immediate advantage compared with IFN-γ as biomarkers for that purpose, our data support their relevance in the context of an integrated antiviral immune response. It is unclear if their performance varies in subsequent viral replication episodes, such as may be frequently seen in D+/R− patients following the primary replication event. One limitation of our study was that we assessed only cytokines and chemokines present in acellular plasma samples, and many other potentially relevant IFN-γ-regulated surface receptors and intracellular molecules not tested in the present work may still prove useful as biomarkers.

Connecting the ex vivo and in vivo importance of CCL8 in CMV infection outcomes is the association between the promoter SNP rs3138035 and susceptibility to CMV viremia in D+/R− patients. Serostatus-based risk stratification (i.e., D+/R−, D+/R+, D−/R+, and D−/R−) hides a substantial heterogeneity of the individual risks for CMV replication and progression to CMV disease. For example, the risk of CMV disease among D+/R− patients in the first year posttransplant ranged from 6.4 to 58.3% when the organ recipients were regrouped according to CMV-specific cell-mediated immunity testing results at the end of antiviral prophylaxis (4). Tests and markers capable of individualizing the risk of CMV replication may significantly improve CMV preventative strategies. Our data suggest that the CCL8 promoter polymorphism rs3138035 may be a potential candidate for that purpose. This single-nucleotide polymorphism (C/T) also has been shown to be associated with clinical outcomes in non-small cell lung cancer (41). The minor allele (T) frequency is lowest in Africans (8%) and Asians (9%), intermediate in Americans (22%) and highest in Europeans (40%) (1000 Genomes Project phase 1 [42]). This SNP is flanked upstream by the transcriptional insulator CTCF, which is itself up-regulated by CMV infection and plays a repressor role in CMV major promoter gene expression (43). CTCF is responsive to IFN-γ (44), and may be destabilized by downstream promoter sequences (45). It is therefore likely that the rs3138035 TT genotype exhibits distinct responses to both CTCF-mediated CMV repression and IFN-γ-triggered CCL8 induction in monocytes, culminating in reduced CCL8 expression levels. This may explain the CCL8 SNP association with CMV viremia seen in our patients. Additional data suggestive of the relevance of CCL8 to CMV outcomes are the polymorphisms associated with its preferred receptor, CCR5. In recipients of allogeneic stem cell transplants, CCR5 promoter SNPs rs2734648 and rs1800023 were associated with an increased risk of CMV disease (46). Further, rs1800023 was associated with high CMV viral loads in tracheal aspirates of nonimmunosuppressed critically ill patients with CMV reactivation (47). The finding of increased risk of viral replication in rs3138035 TT genotype D+/R− patients seen in the present work merits prospective validation in larger cohorts of patients, as well as elucidation of the mechanisms involved and of potential correlations of this SNP with the kinetics of viral replication.

One important potential implication of our data is the utilization of CCL8 as an immunoadjuvant in CMV vaccines, with the goal of concurrently triggering robust adaptive cellular and humoral responses. CMV vaccine candidates reaching phase 2 clinical trials have achieved some success in stimulating either cellular or humoral responses, but not both simultaneously (6). Chemokines have been explored as a new class of vaccine adjuvant, and have already been tested in animal models for hepatitis C (48), malaria (49), and HIV (50,51). This strategy has already been specifically validated for a CCR5-targeting chemokine-antigen fusion DNA vaccine in mice, which resulted in enhanced CD4+ T cell, CD8+ T cell, and B cell responses (52). Theoretically, intradermal codelivery of immunoadjuvant CCL8 and CMV antigen-coding DNA plasmids should result in priming of naïve and induction of memory Th1 CD4+ T cells by CD1a+ dendritic cells (53), simultaneously yielding Langerhans cells capable of attracting naïve CD8+ T cells to secondary lymph nodes for priming (54,55). To this end, priming efficiency for CMV-specific CD8+ T cell clones may potentially increase when dendritic cells expressing both CCL8 and CMV antigens (56) recruit CCR5+ helper polyclonal CD8+ T cells. Memory CD8+ T cells could also arise from such interactions with dendritic cells, contingent on help from CD4+ T cells (57). CCR5 is also expressed by naïve B cells (58,59), thereby allowing for their interaction with CD14+ dermal dendritic cells expressing CMV antigens and CCL8 for B cell priming (60). Immunization of CMV-seropositive individuals may be especially challenging, given the multiple viral immune evasion mechanisms expressed in infected cells. In this context, CCL8 is a particularly attractive choice of immunoadjuvant since it is spared by the scavenging activity of virally-encoded chemokine receptors in CMV-infected cells (61), and since it promotes preferential naïve CD8+ CCR5+ T cell priming by dendritic cells, in lieu of virus-infected macrophages, in draining lymph nodes (55).

In summary, our findings suggest that the chemokine CCL8 is important to controlling CMV replication in transplant recipients. This knowledge could potentially be translated into personalized monitoring and prophylactic strategies for patients at increased risk for CMV replication, and contribute to the improvement of candidate CMV vaccines.

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Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. DK has received honoraria as a consultant to and received research grant from Roche. AH has received grant support from Roche and honoraria as a consultant to Astellas and Novartis. LFL, AE, JF, DO, OM, and SH have no conflicts of interest to disclose.

References


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Supporting Information

Additional Supporting Information may be found in the online version of this article.
Table S1: Quantitation yields of cytokines and chemokines within the linear range of detection in QuantiFERON CMV and NIL samples from 37 CMV-viremic transplant recipients.

Table S2: Overall cytokine and chemokine responses of 37 CMV-viremic transplant recipients after whole blood incubation in QuantiFERON CMV and NIL tubes.

Table S3: Outcome-specific statistically significant cytokine and chemokine responses of 37 CMV-viremic transplant recipients after whole blood incubation in QuantiFERON CMV and NIL tubes.

Table S4: Outcome-specific nonstatistically significant cytokine and chemokine responses of 37 CMV-viremic transplant recipients after whole blood incubation in QuantiFERON CMV and NIL tubes.

Figure S1: Hierarchical gating strategy for fluorescence-activated cell sorting of lymphocytes. Cells with forward (FSC-A) and side scatter (SSC-A) characteristics compatible with lymphocyte populations were initially selected (top left). Single (top right and middle left) live (middle right) cells were then separated according to expression of the markers CD19 (B cells) (bottom left), CD3+/CD4+ (T-helper) and CD3+/CD8+ (T-cytotoxic) (bottom right).

Figure S2: Hierarchical gating strategy for fluorescence-activated cell sorting of monocytes and NK cells. A nonrestrictive gate containing all PBMC subpopulations (top left) was initially selected. Only live cells (top right; leftmost peak with the lowest amine-reactive dye staining) proceeded to a B and T cell exclusion step (i.e. selection of CD3-/CD19- cells) (bottom left) prior to sorting according to expression of the markers CD56+/CD14- (NK cells) and CD14+ (monocytic cells).