Original Article

Innate Immunity
0(0) 1–10
© The Author(s) 2018
Reprints and permissions:
sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/1753425918757898
journals.sagepub.com/home/ini
SAGE

Characterization of innate immune viral sensors in patients following allogeneic hematopoietic stem cell transplantation

Sarah L Caddy[1], Meng Wang[2], Pramila Krishnamurthy[2], Benjamin Uttenthal[2], Anita Chandra[3], Charles Crawley[2] and Leo C James[1]

Abstract

Viral infection is a major cause of morbidity and mortality following allogeneic hematopoietic stem cell transplant (HSCT), with up to one in four deaths directly linked to viral disease. Whilst awaiting lymphocyte reconstitution post-HSCT, the innate antiviral immune response is the first line of defense against invading viruses. Several novel innate viral-sensing pathways have recently been characterized, but their physiological importance in humans is poorly understood. We analyzed a panel of innate viral-sensor genes in HSCT patients, and assessed whether differences in innate antiviral responses could account for variation in susceptibility to viral infections. Expression levels of innate viral sensors in HSCT patients with active viral infections, HSCT patients without active infections and healthy volunteers were highly homogenous. Although IFN-α expression was up-regulated in actively infected patients relative to controls, a corresponding up-regulation of innate viral sensor expression was not observed. IFN-α stimulation of patient PBMCs *in vitro* showed intact IFN-α signaling, but actively infected patients' PBMCs had reduced up-regulation of innate viral sensors. We show that the aberrant IFN-α responses in HSCT patients were not due to calcineurin inhibition. Our data therefore raises the possibility of an intrinsic defect in innate viral sensor up-regulation in HSCT patients following viral infection.

Keywords

Hematopoetic stem cell transplant, innate immune sensor, virus

Date received: 29 August 2017; revised: 22 December 2017; accepted: 16 January 2018

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an established treatment for hematological malignancies and certain bone marrow defects. All patients receiving allogeneic HSCT experience a prolonged period of compromised immunity, owing to the long time (up to 1 yr) taken for reconstitution of a functional lymphocyte population by the donor bone marrow, combined with concurrent use of immunosuppressive drugs that further suppress T-lymphocyte activity. Studies commonly report virus infections to occur in up to around 40% patients post-HSCT in both the pediatric and adult setting,[1–4] with reported mortality ranging from 10% to 50%.[4] A significant proportion of viral infections are caused by reactivation of herpesviruses [predominantly cytomegalovirus (CMV) and Epstein–Barr virus (EBV)]. Infection by adenovirus and numerous respiratory viruses also contribute to poor patient outcome.[1]

A number of risk factors have been linked to increased viral infection and higher transplant-related

[1]MRC Laboratory of Molecular Biology, Cambridge, UK
[2]Department of Haematology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK
[3]Department of Immunology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

Corresponding author:
Sarah L Caddy, MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, UK.
Email: slc50@cam.ac.uk

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (http://www.creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).

mortality in patients following allogeneic HSCT. Donor selection plays an important role. For example, mismatch between recipient and donor CMV serostatus has been associated with increased infections, and a higher transplant-related mortality in unrelated allogeneic HSCT.[5,6] Recipient and treatment characteristics that delay or impair immune reconstitution also increase the risk of viral infections post-HSCT.[7] These factors include recipient age and comorbidity, significant pretreatment immunosuppression, e.g. fludarabine, higher conditioning intensity, T-cell-depletion strategies, e.g. alemtuzumab, graft versus host disease (GvHD) and immunosuppression after the transplant. However, these risk factors do not allow accurate prediction of all HSCT patients that succumb to viral infection, suggestive of additional undefined variation between patients.

Over the past decade, many intracellular antiviral pathways intrinsic to all mammalian cells have been elucidated. These include identification of several novel mechanisms of detecting the invading virus in the host cell cytoplasm. For viruses commonly infecting HSCT patients, the innate immune recognition pathways of particular interest are the cGAS-STING axis capable of recognizing double-stranded DNA from human herpesviruses and adenovirus,[8–10] the RIG-I pathway for detection of viral double-stranded RNA and 5'PPP-RNA from viruses such as human influenza and parainfluenza,[11,12] and the intracellular immunoglobulin receptor TRIM21, which identifies invading viruses tagged by Abs.[13] The antiviral sensing pathways are known to be up-regulated by IFN-α, and a major downstream effector mechanism from the viral sensors is to further increase the expression of IFN-α, thus forming a positive-feedback loop during a viral infection.[14] In conjunction with up-regulation of these viral sensors, IFN-α also induces the expression of hundreds of genes collectively known as IFN-stimulated genes (ISGs) that allow both virally infected and bystander cells to mount an antiviral immune response.[15]

The role of the many innate viral sensors has been extensively characterized *in vitro* and in animal models. For example, mice deficient in cGAS, RIG-I or TRIM21 rapidly succumb to viral infections,[10,16–18] showing these innate sensor mechanisms play a vital role in defense against virus infections. However, in humans the relative contribution of many of these ISGs compared with adaptive immune responses against virus infections is poorly understood, with few ISGs so far shown to control virus infections in humans.[14]

Following HSCT, monocytes play an important early role in protection against infection. Their reconstitution is faster than lymphocytes (30 d vs. up to 1 yr), and a delay in monocyte recovery has been associated with an increased frequency of infection complications, as well as a poorer overall survival rate.[19,20] Activation of monocytes in the face of viral infection first requires recognition of the invading pathogen via the innate immune sensors. We hypothesize that dysregulation of the innate immune virus sensors could account for variation in susceptibility to virus infection following HSCT treatment.

To this end, we assessed the innate viral sensing pathways and cytokine profile produced by monocytes in HSCT patients with and without active viral infections, as well as healthy volunteers. Our results did not show a consistent relationship between up-regulation of IFN-α and expression of the viral sensors in HSCT patients. Intact IFN-α signaling pathways were demonstrated in patient PBMCs and shown not to be affected by immunosuppressive therapy, but actively infected patients had reduced up-regulation of the innate viral sensors vs. patients without active infections or healthy volunteers. Our study provides the first insight into the intracellular antiviral immune responses of patients that have received HSCT, and shows IFN-α signaling to be heterogeneous and complex in this patient cohort.

Materials and methods

Patient recruitment and sample collection

Peripheral whole-blood samples were collected into EDTA tubes from both patients and healthy volunteer adult donors. Ethical consent for the study was approved by the Research Ethics Committees for each donor group separately; HSCT patients (REC 07/MRE05/44), patients with primary immunodeficiency receiving regular immunoglobulin therapy ('PI patients') (REC 12/WA/0148); and healthy volunteers (16/LO/0997). All participants were older than 18 yr and gave written informed consent. Samples were anonymized at point of collection by the phlebotomist. Patients were recruited from Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust, and healthy volunteers were recruited from the Medical Research Council Laboratory of Molecular Biology. All HSCT patients recruited were within 1 yr of receiving the HSCT. PI patients were recruited if they were receiving immunoglobulin therapy, either intravenously or subcutaneously for 1 mo or longer. Leukocyte differential counts were performed on a Siemens ADVIA2500i platform by the Department of Haematology clinical laboratory at Addenbrooke's Hospital.

Transplantation protocols

The transplantation protocols were approved by the local research ethics committee and informed consent was obtained from all patients. Human leukocyte Ag (HLA) typing of recipients and donors was carried out by high-resolution molecular techniques. The selection of conditioning regimen and source of hematopoietic

stem cells were based on hematological diagnosis, donor availability and clinical state of the patient. GvHD prophylaxis for standard conditioning regimens consisted of lymphodepletion by alemtuzumab or antithymocyte globulin (ATG), or methotrexate in the conditioning regimen, with a calcineurin inhibitor (ciclosporin/tacrolimus) administered for a minimum of 6 mo and tapered thereafter. In reduced-intensity conditioning regimens, the GvHD prophylaxis was lymphodepletion by alemtuzumab ATG, and a calcineurin inhibitor, administered from d 1 and tapered from d 56 for sibling transplants and d 70 for matched unrelated transplants in the absence of GvHD. For haploidentical transplants, the GvHD prophylaxis consisted of cyclophosphamide, mycophenolate mofetil and tacrolimus. Chimerism analysis was performed routinely on d 28, 56, 100, then 6 mo after HSCT using SNP analysis. Red cells and platelets were transfused to maintain hemoglobin level and platelet count above 80 g/l and 10×10^9/l, respectively. All patients received antiviral and antifungal prophylaxis using aciclovir and a triazole agent or AmBisome. Prophylaxis with azithromycin was given to donors or recipients with serological evidence of toxoplasma exposure. All patients were monitored weekly for CMV, EBV and adenovirus DNA. Monitoring for additional viruses, e.g. respiratory viruses, BK, JC and norovirus, was carried out based on patient clinical status.

Isolation, storage and fractionation of PBMCs

PBMCs were purified from whole-blood samples using the standard Ficoll gradient technique within 4 h of collection. Briefly, 20 ml whole blood was layered over 20 ml Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 600 *g* for 30 min. The PBMC interface was carefully removed by pipetting and washed with PBS by centrifugation at 300 *g* for 10 min. PBMCs were counted and 1×10^7 cells were cryopreserved in 10% DMSO and 40% FCS. $CD14^+$ monocytes were enriched from the remaining PBMCs using magnetic-activated cell sorting (MACS MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Monocyte enrichment was necessary because flow cytometry data (data not shown) revealed variability in leukocyte differential composition between HSCT patients, and preliminary results showed variation in gene expression between $CD14^+$ and $CD14^-$ cells.

RNA extraction and quantitative real-time PCR

RNA was extracted from freshly isolated $CD14^+$ monocytes using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The yield and purity of RNA were determined by spectrophotometric measurements of the ratio of UV absorbance at 260 and 280 nm by a Nano-drop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was then stored at –80°C until use. RNA was extracted from $CD14^+$ monocytes of each subject at a concentration >20 ng/μl.

$CD14^+$ monocyte RNA was reverse transcribed using Super RT reverse transcriptase (HT Biotechnology, Cambridge, UK). cDNA was then used for quantitative PCR (qPCR) on an AB Biosystems StepOnePlus machine with a panel of Taqman-based primer probes (Thermo Fisher Scientific) with the following cycling conditions for 40 cycles: 95°C for 1 s, 60°C for 20 s. Following analysis of four widely used house-keeping genes (β-actin, *HPRT*, *TBP* and *RPLP0*), the most stable house-keeping gene in samples obtained from healthy volunteers was determined to be β-actin. Gene amplifications were therefore normalized to β-actin and subsequently the delta-delta C_T method of analysis was conducted with reference to gene expression in the healthy volunteer samples. Only patients with detectable IFN-α levels were compared by the delta-delta C_T method of analysis to healthy volunteers.

In vitro *stimulation of PBMCs*

Cryopreserved PBMCs from patients and healthy controls were thawed, washed, plated at 0.5×10^6/well and rested overnight at 37°C in RPMI, supplemented with 10% FCS and 250 U/ml penicillin, and 250 μg/ml streptomycin. Cells were then stimulated with 1000 IU IFN-α or PBS control for 6 h. Where indicated, tacrolimus (LKT Laboratories, St. Paul, MN, USA) was added to cells 2 h prior to IFN-α stimulation at 10-100 ng/ml. Following stimulation, cells were harvested for RNA extraction and quantitative real-time PCR (qRT-PCR) as previously described.

Statistics

P-Values were calculated using the χ^2test.

Results

Patient characteristics

Whole-blood samples were collected from 15 patients that had received HSCT between February 2015 and September 2016 at Addenbrooke's Hospital, Cambridge University Hospitals NHS Foundation Trust (Table 1). Each patient was sampled at a single time point between d 30 and 339 after their transplant. Ten of these patients had an active viral infection confirmed by detection of viral nucleic acid. Of the infected patients, four were infected with CMV, two with human norovirus, two with BK virus, one with CMV and BK virus, and one with parainfluenza virus genotype 3. Two patients had chronic viral infections

Table 1. Clinical and laboratory characteristics of patients recruited post-HSCT.

		Virus infection	No virus infection	*P*-Value
No of patients		10	5	
Sex	M	4	3	0.29
Average age at HSCT		55 (15)	54 (13)	0.93
Diagnosis	AML	5	2	NS
	MDS	0	3	
	NHL	4	0	
	ALL	1	0	
HSCT type	MUD	6	4	0.62
	Sib Allo	2	1	
	Haplo	2	0	
d from HSCT		79 (63)	218 (119)	0.01
Source of stem cells	Peripheral blood	10	5	NA
Condition regimen	RIC	9	5	0.64
	Myeloablative	1	0	
Fludarabine-based conditioning	Yes	9	5	1.00
	No	1	0	
Lymphodepletion (Campath/ATG)	Yes	8	4	1.00
	No	2	1	
HLA mismatch	0	6	5	0.15
	1	2	0	
	2	1	0	
	>2	1	0	
Sex mismatch	No	6	3	0.76
	Mismatch	4	1	
	ND	0	1	
Concurrent immunosuppression	Yes	9	3	0.17
	No	1	2	
GvHD	Yes	6	3	1
	No	3	2	
	ND	1	0	
CMV serostatus (donor/recipient)	Pos/pos	6	0	0.07
	Neg/neg	1	1	
	Neg/pos	0	4	
	Pos/Neg	3	0	
EBV serostatus (donor/recipient)	Pos/pos	10	5	NA
HIV/HepB/HepC	Neg/neg/neg	10	5	NA
White cell count ($\times 10^6$/ml)	Total	3.85 (2.27)	6.04 (2.73)	0.12
	Monocyte	0.32 (0.17)	0.69 (0.48)	0.007
	Lymphocyte	0.62 (0.59)	0.84 (0.50)	0.47

SD is presented in brackets after the mean value, where appropriate. *P*-values are calculated using the χ^2 test as indicated. HepB: hepatitis B; HepC: hepatitis C; M: male; AML: Acute myeloid leukaemia; MDS: Myelodysplastic syndrome; NHL: Non-Hodgkin lymphoma; ALL: Acute lymphoblastic leukaemia; MUD: matched unrelated donor; Sib Allo: sibling allotype; Haplo: haplotype; NS: not significant; NA: not applicable; RIC: reduced intensity conditioning; ND: not determined; Pos: positive; Neg: negative.

detectable for > 100 d (one patient infected with norovirus and one patient infected with BK virus), whereas the remaining eight actively infected patients had a mean duration of infection of 23.6 d. To ensure as much as possible that sample collection coincided with the viral infection, blood was taken within 7 d of the most recent positive viral nucleic acid assay and corresponding clinical symptoms if present. HSCT

patients were defined as not having an active virus infection if they exhibited no clinical symptoms suggestive of viral infections, and had a negative viral nucleic acid assay no more than 30 d from sample collection. Samples from five healthy volunteers were also collected.

Several clinical characteristics known to increase susceptibility to viral infections were equally represented in infected and uninfected HSCT patients: HSCT type, myeloablative or reduced intensity conditioning, use of lymphodepletion and fludarabine in the conditioning regimen, concurrent immunosuppression, GvHD and CMV serostatus mismatch. However, a significant difference in monocyte counts between infected (0.32×10^9/l) and uninfected (0.69×10^9/l) patients was identified ($P = 0.0068$), with lower monocyte counts apparent in the infected cohort (Table 1). To avoid bias introduced by the difference in cell counts between the different patient groups, $CD14^+$ monocytes were enriched from the peripheral blood sample by cell sorting prior to RNA extraction and analysis of gene expression. Another difference between our HSCT patient cohorts was the time of sample collection following HSCT; at a median d 78 post-HSCT for infected and median d 218 for uninfected HSCT patients. This likely accounts the increased monocyte counts in the uninfected HSCT patient cohort given the longer time interval for immune reconstitution.

Up-regulation of IFN-α in monocytes of patients with active viral infections

We assessed expression of the major antiviral cytokine IFN-α in monocytes isolated from whole-blood samples. IFN-α expression was detected in 6/10 actively infected HSCT patients but only 1/5 HSCT control patients and 1/5 of the healthy volunteers (Figure 1). In addition, the IFN-α level in the actively infected HSCT patients trended higher than control patients and healthy volunteers, although this did not achieve statistical significance. Increased detection of IFN-α in actively infected patients is in agreement with reports that have examined IFN-α expression in the context of a wide range of virus infections, from HIV to human norovirus;[21,22] IFN-α expression is up-regulated by cells following virus recognition.

Minimal variation in expression of immune sensors by monocytes between patients and volunteers

The innate viral sensors have been shown to be up-regulated in response to IFN-α during viral infections in cell line and murine experiments.[23] Having identified a trend toward up-regulation of IFN-α in virally infected HSCT patients relative to uninfected patients and healthy volunteers, we predicted a corresponding increase in the expression of a panel of innate

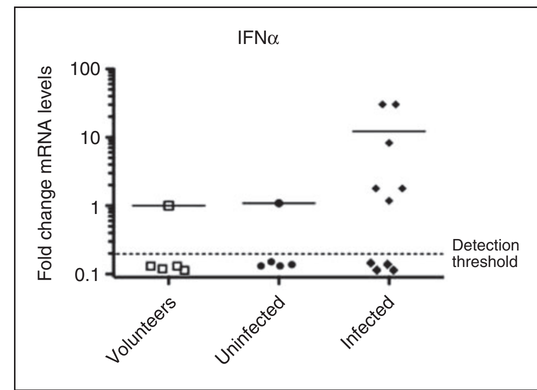

Figure 1. Gene expression of IFN-α in $CD14^+$ monocytes by qRT-PCR from HSCT patients with and without virus infections. All data were normalized to β-actin (*ACTB*) RNA and healthy volunteers by the delta-delta C_T method of analysis. The line for each group represents the mean.

viral sensors and signaling adaptors (RIG-I, cGAS, IFIT1, TLR9, TRIM21, AIM2, STING and MYD88). However, we found these genes to be consistently expressed amongst HSCT patients and healthy volunteers with no significant increased expression in virally infected HSCT patients (Figure 2). RIG-I, TLR9 and IFIT1 exhibited a trend toward increased expression (but was not statistically significant) in HSCT patients vs. healthy volunteers, but there was no obvious difference between HSCT patients with or without active infections. The innate immune sensors each detect different classes of virus; thus, we wanted to assess whether sub-group analysis of the virally infected patients by virus type would alter the results. We compared gene expression in individuals infected with DNA viruses (detected by cGAS, AIM2 and TLR9) vs. RNA viruses (detected by RIG-I), and enveloped vs. non-enveloped viruses (TRIM21 detects non-enveloped viruses via bound Abs[13]). However, we did not identify any obvious separation in gene expression levels using these sub-group analyses (data not shown). A major limiting factor here was the low number of patients in the sub-groups. Overall, the innate immune sensors and their signaling adaptors were expressed equally in monocytes from HSCT patients and healthy volunteers with no obvious up-regulation in the monocytes of HSCT patient with active viral infections despite increased IFN-α expression.

Variable responsiveness to IFN-α stimulation of PBMCs in vitro

To determine whether the lack of innate immune sensor up-regulation in virally infected patients was due to an abnormality in their ability to respond to IFN-α, we performed *in vitro* stimulation experiments. PBMCs from a subset of HSCT patients with active viral infections and PBMCs from three healthy volunteers were

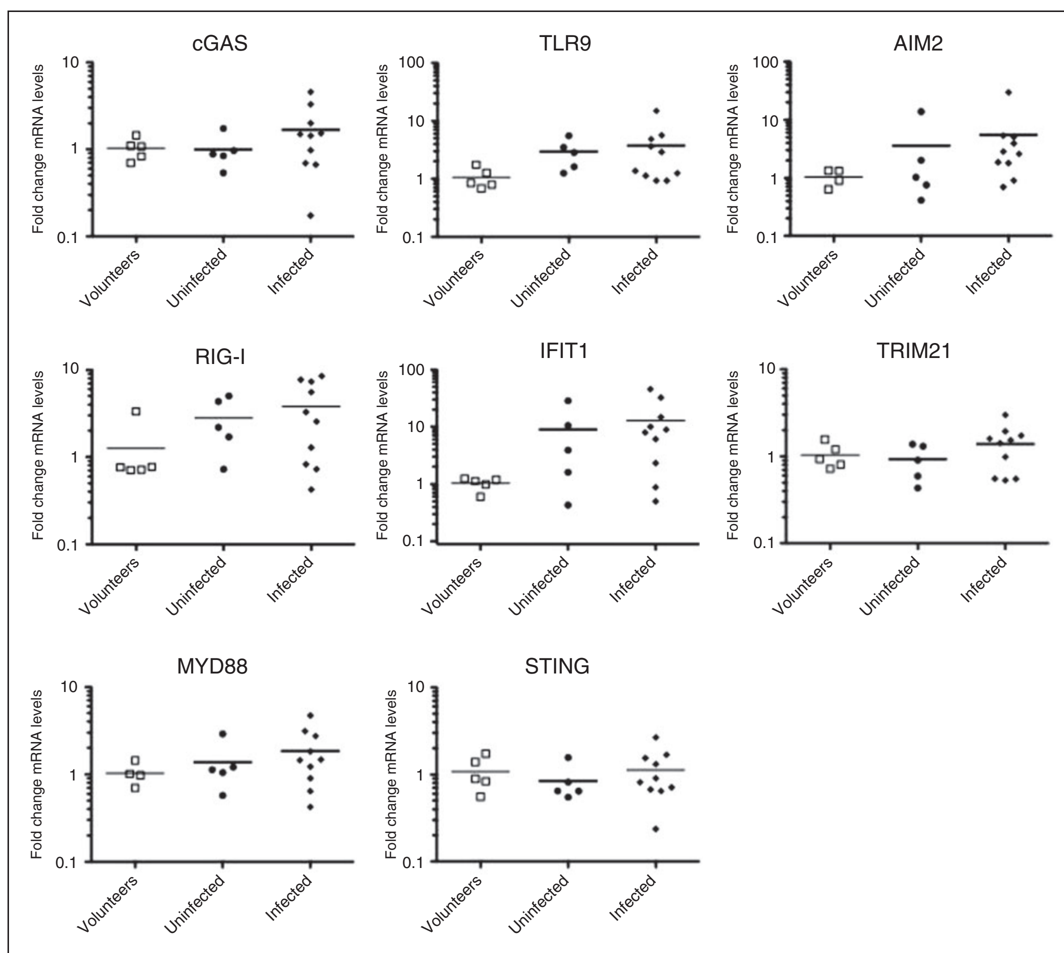

Figure 2. Gene expression of innate immune viral sensors and adaptors. Gene expression was analyzed by qRT-PCR of CD14^{+} monocytes from HSCT patients with and without virus infections. All data were normaliszed to β-actin (*ACTB*) RNA and healthy volunteers by the delta-delta Ct method of analysis. The line for each group represents the mean.

recovered following cryopreservation, allowed to recover overnight in culture and then stimulated with high-dose IFN-α (1000 IU) *in vitro* for 6 h. RNA was extracted from stimulated PBMCs and gene expression of innate immune sensors analyzed by qRT-PCR as previously described.

We initially assessed for up-regulation of CXCL10 and IFIT1, which are known to be strongly induced by IFN-α.[24,25] This was, indeed, what we observed, with PBMCs from actively infected HSCT patients and healthy volunteers exhibiting an average 350-fold increase in expression of CXCL10 and IFIT1 in response to IFN-α (Figure 3a). This suggests that PBMCs from HSCT patients are sensitive to IFN-α, and validates our *in vitro* experimental approach. We then proceeded to assess TRIM21, RIG-I and cGAS expression in the same experimental system (Figure 3b). Whereas IFN-α-stimulated PBMCs from healthy volunteers up-regulated TRIM21 (average 3.5-fold increase) and RIG-I (average 23-fold increase), PBMCs from actively infected HSCT patients failed to up-regulate TRIM21 or RIG-I, despite exogenous IFN-α stimulation, vs. healthy volunteers (TRIM21: $P = 0.0016$; RIG-I: $P = 0.0001$). The same trend was observed for cGAS up-regulation of expression in response to IFN-α stimulation; a 30-fold increase was observed in healthy volunteers vs. only a twofold increase in HSCT patients with active viral infections (difference not statistically significant). This reduced up-regulation of innate immune sensors is not due to a global insensitivity to IFN-α since the HSCT patient PBMCs were able to up-regulate CXCL10 and IFIT1.

Evaluation of effect of immunosuppression on response to IFN-α

A suggested cause for the lack of up-regulation of the innate immune receptors in HSCT patient monocytes and *in vitro*-stimulated PBMCs could be due to the immunosuppressive therapy regimens altering IFN-α signaling pathways.[26,27] At the time of sample collection, 7/10 actively infected HSCT patients were receiving calcineurin inhibitors (tacrolimus/ciclosporin), and 2/10 of these patients were also

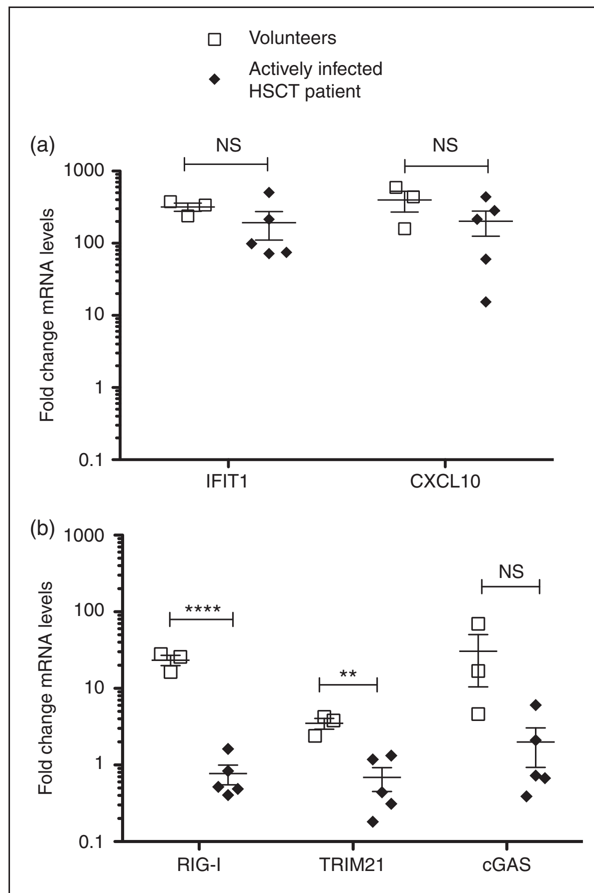

Figure 3. Gene expression of ISG transcripts of PBMCs isolated from HSCT patients stimulated by IFN-α *in vitro*. All data were normalized to β-actin (*ACTB*) RNA and un-stimulated patient PBMCs by the delta-delta Ct method of analysis. (a) Highly inducible ISGs and (b) innate immune sensor genes. Volunteers $n = 3$; actively infected HSCT patients $n = 5$. Experiments carried out in three technical replicates. Error bars = SE. ***P*0.001–0.01, ****P*0.001–0.0001. NS: not significant.

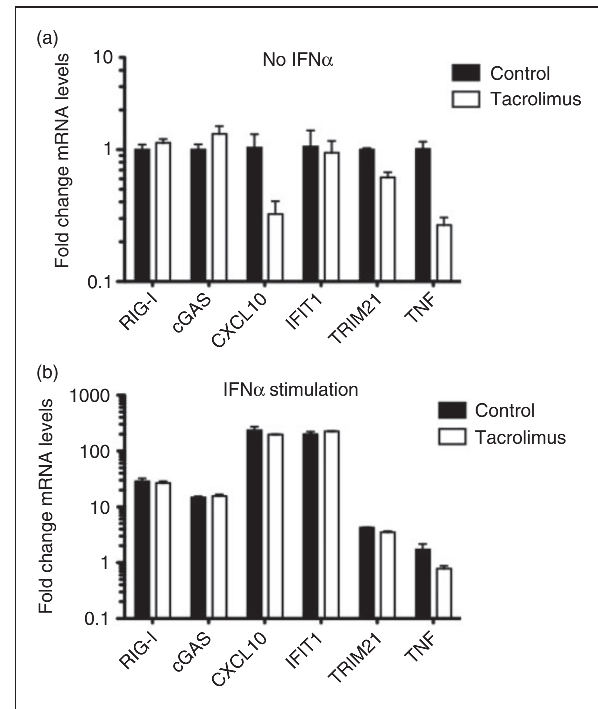

Figure 4. Effect of tacrolimus on ISG expression in (a) unstimulated PBMCs and in (b) PBMCs stimulated with IFN-α.

receiving methylprednisolone for GvHD. Two of five HSCT patients with no active infection were receiving tacrolimus and 1/5 receiving prednisolone. In addition, PBMCs used for the *in vitro* stimulation experiments were all from HSCT patients that were receiving a calcineurin inhibitor. To investigate the possibility that calcineurin inhibitors could account for the discrepancy in innate virus sensor expression pattern in HSCT patients, we cultured PBMCs from healthy volunteers in the presence or absence of tacrolimus. The target therapeutic range for whole-blood tacrolimus in patients was between 5 and 15 ng/ml (median 10 ng/ml); therefore, PBMCs were incubated with or without 10 ng/ml tacrolimus for 2 h prior to stimulation with 1000 IU IFN-α or the equivalent volume of PBS for 4 h. RNA was then extracted from PBMCs and gene expression analyzed by qRT-PCR.

In IFN-α-unstimulated PBMCs, tacrolimus treatment resulted in down-regulation of CXCL10 and TNF-α by 50%, but had no effect on the expression level of the innate immune receptors cGAS, RIG-I and TRIM21 (Figure 4a). In response to IFN-α stimulation, we observed robust up-regulation of ISGs, including cGAS, RIG-I and TRIM21, which was not affected by tacrolimus treatment (Figure 4b). This shows that in PBMCs, innate virus sensor induction by IFN-α is unaffected by tacrolimus.

PBMCs from virally infected primary immunodeficiency patients up-regulate innate viral sensors

We wanted to assess whether the loss of IFN-α-induced up-regulation of innate immune sensors in HSCT patients with active viral infections was specific to the HSCT treatment or a consequence of viral infection. To investigate this, we tested PBMCs from five patients with primary immunodeficiency that required long-term intravenous immunoglobulin or subcutaneous immunoglobulin therapy for recurrent viral infections ('PI patients'). All five PI patients had confirmed active viral infections; one norovirus, two parainfluenza, and two rhinovirus infections.

PBMCs isolated from PI patients with active infections retained the ability to up-regulate CXCL10, IFIT1, RIG-I, TRIM21 and cGAS in response to *in vitro* IFN-α stimulation (Figure 5). Although RIG-I was expressed at lower levels in PBMCs from PI patients with active viral infections following IFN-α stimulation, this still amounted to a 10-fold up-regulation of expression vs. unstimulated PBMCs (depicted by the horizontal line at y = 1). This is in contrast to the

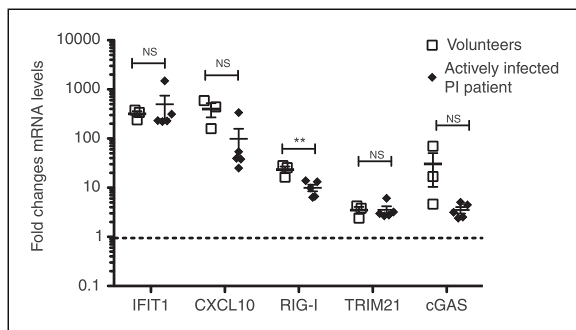

Figure 5. Analysis of effect of virus infection on ISG expression in patients with primary immunodeficiency (PI). Gene expression was analyzed by qRT-PCR in patients with and without active virus infections in PBMCs stimulated by IFN-α *in vitro*. All data were normalized to β-actin (*ACTB*) RNA and un-stimulated patient PBMCs by the delta-delta C_T method of analysis. Volunteers $n = 3$; actively infected primary immunodeficiency patients $n = 5$. Dotted horizontal line at $y = 1$ corresponds to no change in mRNA expression relative to unstimulated PBMCs. $^{**}P = 0.001–0.01$. NS: not significant.

PBMCs from actively infected HSCT patients, where the expression of RIG-I and TRIM21 did not change in response to IFN-α stimulation, and cGAS expression was only up-regulated twofold.

Discussion

Virus infections are a common cause of mortality in allogenic HSCT patients, but risk factors that increase the likelihood of succumbing to virus infection in these patients are poorly defined. Post-HSCT, the adaptive immune response is severely impaired owing to the time taken to reconstitute a functional lymphocyte population and the use of immunosuppressive therapies. Our HSCT patient population has several risk factors for viral infections, including the use of fludarabine conditioning regimens and lymphodepletion with alemtuzumab or ATG, both contributing to a delayed immune reconstitution. Therefore, the innate immune system must play an important role in protecting patients from virus infections during this period. Several new innate immune viral sensors have recently been identified, but their importance in human populations has not been evaluated in detail. We therefore wanted to characterize the expression of the newly discovered viral sensors in HSCT patients, and assess whether differences between virally infected and uninfected patients could contribute to susceptibility to virus infections.

We first analyzed gene expression in $CD14^+$ monocytes from 10 virally infected HSCT patients, five uninfected HSCT patients and five healthy volunteers. We observed a trend towards increased IFN-α expression in monocytes from virally infected HSCT patients. However, this did not translate to increased ISG expression, with the innate immune viral sensors cGAS, RIG-I, TRIM21, IFIT1 and AIM2, and the adaptor proteins MYD88 and STING, expressed at similar levels between infected and uninfected HSCT patients, and healthy volunteers. We proceeded to functionally test the ability of HSCT patient PBMCs to respond to IFN-α stimulation. We found that PBMCs from virally infected HSCT patients were unable to up-regulate the innate immune viral sensors RIG-I and TRIM21 in response to IFN-α. Thus, the *in vitro* data mirror the *in vivo* finding from HSCT patient monocytes. This is despite using a mixed-cell population of PBMCs for the *in vitro* stimulation experiments (due to sample availability), but suggests a possible global defect in the IFN-α immune response across multiple cell types. We have shown that this apparent insensitivity to IFN-α was not due to the effect of calcineurin inhibitors as treating healthy volunteer PBMCs with tacrolimus did not inhibit up-regulation of the ISGs following IFN-α stimulation. PBMCs from virally infected patients with primary immunodeficiency exhibited IFN-α sensitivity with ISG up-regulation in a similar manner to healthy volunteers, suggesting the apparent failure to up-regulate the innate immune viral sensor genes is specific to HSCT patients.

To the best of our knowledge, this is the first time that expression of these innate immune viral sensors have been analyzed in PBMCs from HSCT patients. Our findings demonstrate that these genes are actively expressed and detectable. However, the failure of actively infected HSCT patient monocytes and PBMCs to up-regulate the innate immune viral sensors despite IFN-α stimulation was unexpected. This did not represent a global deficiency in the cellular response to IFN-α, as CXCL10 and IFIT1 that are known to be strongly up-regulated in response to IFN-α were indeed up-regulated in PBMCs from all patients examined.

What might be the cause for this apparent defective IFN-α response in the immune innate sensors in virally infected HSCT patients? As 7/10 actively infected HSCT patients were receiving calcineurin inhibitors as part of their GvHD prophylaxis, we assessed whether immunosuppression could play a role in suppressing the IFN-α response in the monocytes. Tacrolimus inhibits the phosphorylase activity of calcineurin, which, in turn, inhibits the transcription factor NFAT required for transcriptional activation of IL-2. IL-2 is a key growth factor for T cells; therefore, it has been understood that the immunosuppressive effect of tacrolimus is achieved primarily through T-cell suppression.[27] Nevertheless, a number of recent studies have shown NFAT can be expressed in innate immune cells,[28] and tacrolimus can affect these innate immune cells by changing the expression of immunomodulatory cytokines.[27,29] Our results add support to these observations by showing that tacrolimus reduced the expression of TNF-α and CXCL10 in PBMCs pretreated with

tacrolimus, in line with previous reports.[27,30] However, TNF-α and CXCL10 expressions were still able to be up-regulated by IFN-α stimulation, suggesting IFN-α signaling pathways are independent of the effect of tacrolimus. However, in addition we have shown that tacrolimus does not affect the basal expression levels of the innate immune virus sensors (RIG-I, cGAS, TRIM21 and IFIT1), and that IFN-α signaling pathways are not disrupted by tacrolimus. Therefore, calcineurin inhibition is not the cause of the defective IFN-α response observed in virally infected HSCT patients.

We have also demonstrated that the defective IFN-α response is not simply a consequence of viral infection as PBMCs from primary immunodeficiency patients with viral infections were able to up-regulate innate immune viral sensors. This suggests that the failure to up-regulate the innate viral sensors could be specific to allogeneic HSCT treatment. There have been other studies that have also found a functional defect in monocytes after allogeneic HSCT,[31,32] and epigenetic changes in the regulation of the innate immune system have been suggested as a potential cause.[33] In our study, the infected HSCT patients did have a lower monocyte count than uninfected HSCT patients, which could have contributed to increased susceptibility to viral infections. However, our gene expression analysis used purified monocytes, which would have normalized the difference in monocyte count between infected and uninfected HSCT patients. Samples collected from uninfected HSCT patients were, on average, at a later time point following HSCT than infected HSCT patients (218 d vs. 79 d post-HSCT). Therefore, we cannot exclude the possibility that the defective IFN-α response is transient and only affects the early phase post-HSCT. Our data therefore suggest it is an as-yet-undefined characteristic of actively infected HSCT patients that impairs their ability to up-regulate the innate immune viral sensors in response to IFN-α.

The main limitations of our study were firstly only sampling the patients at a single time point. For actively infected HSCT patients, we are therefore unable to conclude whether the defective IFN-α response preceded the viral infection, or occurred during viral infection, and whether the monocytes regained full IFN-α sensitivity after the resolution of the viral infection. A time course analysis of HSCT patient monocytes will allow us to address these questions, and give further insight into whether such a defective IFN-α response could increase susceptibility to viral infections. Future studies should aim to look at specific immune cell types, and possibly non-immune cell innate viral sensor responses to IFN-α and viral infection in HSCT patients. Lastly, access to a larger cohort of patients would have allowed more detailed characterization of the innate immune response to different viruses, and how different patient and transplant characteristics could have affected the IFN-α response.

To conclude, this study provides a valuable, initial insight into the intracellular antiviral immune responses of HSCT patients. It has been demonstrated that IFN-α signaling is varied and complicated in this patient cohort, and we have provided preliminary evidence that the ability of HSCT patients to up-regulate antiviral ISGs in the face of viral infection is impaired. This could be a useful clinical biomarker to predict susceptibility to viral infections after HSCT, as well as a potential therapeutic opportunity if mechanisms exist to enhance the innate immune viral sensor expression. Therefore, further studies to investigate IFN-α signaling pathways in response to virus infection in clinical patients are warranted.

Acknowledgements

We thank Madelein Crause for sample collection from patients.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by a Junior Research Fellowship to SLC from Magdalene College, Cambridge. This work was supported by the Medical Research Council MC_U105181010.

ORCID iD

Sarah L Caddy http://orcid.org/0000-0002-9790-7420.

References

1. Schuster MG, Cleveland AA, Dubberke ER, et al. Infections in hematopoietic cell transplant recipients: results from the organ transplant infection project, a multicenter, prospective, cohort study. *Open Forum Infect Dis* 2017; 4: 1–7.
2. Hiwarkar P, Gaspar HB, Gilmour K, et al. Impact of viral reactivations in the era of pre-emptive antiviral drug therapy following allogeneic haematopoietic SCT in paediatric recipients. *Bone Marrow Transplant* 2013; 48: 803–808.
3. Lin R and Liu Q. Diagnosis and treatment of viral diseases in recipients of allogeneic hematopoietic stem cell transplantation. *J Hematol Oncol* 2013; 6: 94.
4. Shah DP, Ghantoji SS, Mulanovich VE, et al. Management of respiratory viral infections in hematopoietic cell transplant recipients. *Am J Blood Res* 2012; 2: 203–218.
5. Boeckh M, Nichols WG, Papanicolaou G, et al. Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. *Biol Blood Marrow Transplant* 2003; 9: 543–558.
6. Shaw BE, Mayor NP, Szydlo RM, et al. Recipient/donor HLA and CMV matching in recipients of T-cell-depleted unrelated donor haematopoietic cell transplants. *Bone Marrow Transplant* 2017; 52: 717–725.
7. Tomblyn M, Chiller T, Einsele H, et al. Guidelines for preventing infectious complications among hematopoietic cell transplant recipients: a global perspective recommendations. *Biol Blood Marrow Transplant* 2009; 15: 1143–1238.

8. Paijo J, Döring M, Spanier J, et al. cGAS senses human cytomegalovirus and induces type I interferon responses in human monocyte-derived cells. *PLoS Pathog* 2016; 12: e1005546.
9. Lam E, Stein S and Falck-pedersen E. Adenovirus detection by the cGAS/STING/TBK1 DNA sensing cascade. *J Virol* 2014; 88: 974–981.
10. Li X-D, Wu J, Gao D, et al. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science* 2013; 341: 1390–1393.
11. Kato H, Takeuchi O, Sato S, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006; 441: 101–105.
12. Yoneyama M, Kikuchi M, Natsukawa T, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004; 5: 730–737.
13. Mallery DL, McEwan W, Bidgood SR, et al. Abs mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). *Proc Natl Acad Sci U S A* 2010; 107: 19985–19990.
14. Mcnab F, Mayer-barber K, Sher A, et al. Type I interferons in infectious disease. *Nat Rev Immunol* 2015; 15: 87–103.
15. Schoggins JW and Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* 2011; 1: 519–525.
16. Loo YM and Gale M. Immune signaling by RIG-I-like receptors. *Immunity* 2011; 34: 680–692.
17. Vaysburd M, Watkinson RE, Cooper H, et al. Intracellular Ab receptor TRIM21 prevents fatal viral infection. *Proc Natl Acad Sci U S A* 2013; 110: 12397–12401.
18. Schoggins JW, Macduff DA, Imanaka N, et al. Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* 2014; 505: 691–695.
19. DeCook LJ, Thoma M, Huneke T, et al. Impact of lymphocyte and monocyte recovery on the outcomes of allogeneic hematopoietic SCT with fludarabine and melphalan conditioning. *Bone Marrow Transplant* 2013; 48: 708–714.
20. Storek J, Dawson MA, Storer B, et al. Immune reconstitution after allogeneic marrow transplantation compared with blood stem cell transplantation. *Blood* 2001; 97: 3380–3390.
21. Lehmann C, Harper JM, Taubert D and Hartmann P. Increased interferon alpha expression in circulating plasmacytoid dendritic cells of HIV-1- infected patients. *J Aquir immune Defic Syndr* 2008; 48: 522–530.
22. Newman KL, Moe CL, Kirby AE, et al. Human norovirus infection and the acute serum cytokine response. *Clin Exp Immunol* 2015; 182: 195–203.
23. Schneider WM, Chevillotte MD and Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol* 2014; 32: 513–545.
24. Diamond MS. IFIT1: a dual sensor and effector molecule that detects non-2'-O methylated viral RNA and inhibits its translation. *Cytokine Growth Factor Rev* 2014; 25: 543–550.
25. Groom J and Luster A. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunology* 2011; 89(2): 207–15.
26. Hirano K, Ichikawa T, Nakao K, et al. Differential effects of calcineurin inhibitors, tacrolimus and cyclosporin a, on interferon-induced antiviral protein in human hepatocyte cells. *Liver Transplant* 2008; 14: 292–298.
27. Aomatsu T, Imaeda H, Takahashi K and Fujimoto T. Tacrolimus (FK506) suppresses TNF-α -induced CCL2 (MCP-1) and CXCL10 (IP-10) expression via the inhibition of p38 MAP kinase activation in human colonic myofibroblasts. *Int J Mol Med* 2012; 2: 1152–1158.
28. Fric J, Zelante T, Wong AYW, et al. NFAT control of innate immunity. *Blood* 2012; 120: 1380–1389.
29. Chang KT, Lin HYH, Kuo CH and Hung CH. Tacrolimus suppresses atopic dermatitis-associated cytokines and chemokines in monocytes. *J Microbiol Immunol Infect* 2016; 49: 409–416.
30. Sakuma S, Kato Y, Nishigaki F, et al. FK506 potently inhibits T cell activation induced TNF-a and IL-1b production in vitro by human peripheral blood mononuclear cells. *Br J Pharmacol* 2000; 130: 1655–1663.
31. Levin MJ, Parkman R, Oxman MN, et al. Proliferative and interferon responses by peripheral blood mononuclear cells after bone marrow transplantation in humans. *Infect Immun* 1978; 20: 678–684.
32. Winston DJ, Territo MC, Ho WG, et al. Alveolar macrophage dysfunction in human bone marrow transplant recipients. *Am J Med* 1982; 73: 859–866.
33. Domingo-Gonzalez R and Moore BB. Innate immunity post-hematopoietic stem cell transplantation: focus on epigenetics. *Adv Neuroimmune Biol* 2016; 5: 189–197.