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ORIGINAL ARTICLE

Prophylactic antigen-specific T-cells targeting seven viral and fungal pathogens after allogeneic haemopoietic stem cell transplant

David Jonathan Gottlieb[1,2,3], Leighton Edward Clancy[2,4], Barbara Withers[1,3], Helen Marie McGuire[5,6,7], Fabio Luciani[8], Mandeep Singh[9,10], Brendan Hughes[8], Brian Gloss[3], David Kliman[2], Chun Kei Kris Ma[2], Shyam Panicker[2], David Bishop[1,2,3], Ming-Celine Dubosq[1,2,3], Ziduo Li[3], Selmir Avdic[1,3], Kenneth Micklethwaite[1,2,3,4] & Emily Blyth[1,2,3,4]

[1]Sydney Medical School, University of Sydney, Sydney, NSW, Australia
[2]Blood Transplant and Cell Therapies Program, Westmead Hospital, Sydney, NSW, Australia
[3]Westmead Institute for Medical Research at the University of Sydney, Westmead, NSW, Australia
[4]Sydney Cellular Therapies Laboratory, NSW Health Pathology ICPMR, Sydney, NSW, Australia
[5]Ramaciotti Facility for Human Systems Biology, The University of Sydney, Sydney, NSW, Australia
[6]Charles Perkins Centre, University of Sydney, Sydney, NSW, Australia
[7]Discipline of Pathology, Faculty of Medicine and Health, The University of Sydney, Camperdown, NSW, Australia
[8]Kirby Institute, University of New South Wales, Sydney, NSW, Australia
[9]The Garvan Institute of Medical Research, Darlinghurst, NSW, Australia
[10]Faculty of Medicine, St. Vincent's Clinical School, UNSW, Sydney, NSW, Australia

Correspondence
DJ Gottlieb, Department of Medicine, Level 2 Westmead Hospital, Hawkesbury Rd, Westmead, Sydney, NSW 2145, Australia.
E-mail: david.gottlieb@sydney.edu.au

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Abstract

Objectives. Adoptive immunotherapy using donor-derived antigen-specific T-cells can prevent and treat infection after allogeneic haemopoietic stem cell transplant (HSCT). **Methods.** We treated 11 patients with a prophylactic infusion of 2×10^7 cells per square metre donor-derived T-cells targeting seven infections (six viral and one fungal) following HSCT. Targeted pathogens were cytomegalovirus (CMV), Epstein–Barr virus (EBV), adenovirus, varicella zoster virus, influenza, BK virus (BKV) and *Aspergillus fumigatus*. **Results.** T-cell products were successfully generated in all patients with 10 products responsive to 6 or 7 infections. T-cell infusions were associated with increases in antigen-experienced activated $CD8^+$ T-cells by day 30. CMV, EBV and BKV reactivation occurred in the majority of patients and was well controlled except where glucocorticoids were administered soon after T-cell infusion. Three patients in that circumstance developed CMV tissue infection. No patient required treatment for invasive fungal infection. The most common CMV and EBV TCR clonotypes in the infusion product became the most common clonotypes seen at day 30 post-T-cell infusion. Donors and their recipients were recruited to the study prior to transplant. Grade III/IV graft-versus-host disease developed in four patients. At a median follow-up of 390 days post-transplant, six patients had died, 5 of relapse, and 1 of multi-organ failure. Infection did not contribute to death in any patient. **Conclusion.** Rapid reconstitution of immunity to a broad range of viral and fungal infections can be achieved using a

multi-pathogen-specific T-cell product. The development of GVHD after T-cell infusion suggests that infection-specific T-cell therapy after allogeneic stem cell transplant should be combined with other strategies to reduce graft-versus-host disease.

Keywords: adoptive immunotherapy, allogeneic stem cell transplantation, CTL, haemopoietic stem cell transplant, opportunistic infection, systemic fungal infection, T-cell

INTRODUCTION

It has been almost 30 years since the first demonstration that T-cell immunity to cytomegalovirus (CMV) could be transferred from stem cell donor to recipient via the adoptive transfer of *in vitro*-generated donor-derived CMV-specific T-cells.[1] Since then, many studies have shown that adoptive immunotherapy using antigen-specific T-cells can effectively treat uncontrolled Epstein–Barr virus (EBV) and CMV infections.[2–7] A smaller number of studies have shown that virus-specific T-cells given prophylactically prevent or reduce the severity of post-transplant infections.[8,9]

More recently, adoptive immunotherapy approaches have been extended to the therapy of other viruses including adenovirus, HHV6, BK virus (BKV), Varicella zoster virus (VZV) and Influenza A, all of which cause morbidity and mortality in immunosuppressed transplant recipients.[10–15] Non-viral pathogens are also potential T-cell targets. Using lysates or extracts of common fungal pathogens, we and others have stimulated and expanded T-cells specific for yeasts and moulds.[16–21]

Infection with multiple double stranded DNA viruses is common after allogeneic stem cell transplant (HSCT) and when it occurs is associated with an increased risk of death.[22] T-cells with multiple infectious specificities can be used entirely prophylactically or to enhance immunity to a current infection while simultaneously providing prophylaxis against other infections. Trials of adoptive immunotherapy targeting combinations of CMV, EBV, adenovirus, BK virus and HHV6 and of CMV, EBV, adenovirus and VZV have been reported.[11,23]

In the current trial, a T-cell product specific for CMV, EBV, adenovirus, VZV, BK virus, influenza and *Aspergillus fumigatus* was infused prophylactically. This is the first trial in which seven pathogens have been targeted as part of a prophylactic adoptive immunotherapy strategy and the first that includes a fungal pathogen as a target.

RESULTS

Participant characteristics

Patient characteristics are shown in Table 1. Twelve patients were recruited to the study from November 2013 to December 2015. One patient failed to return for post-transplant or trial follow-up visits and was not included in this analysis. Seven males and four females aged from 26 to 66 years (median 50) were included. All patients underwent transplant for malignant disease. Post-transplant GVHD prophylaxis was with ciclosporin and methotrexate in four cases (2 omitted day 11 methotrexate), ciclosporin and mycophenolate mofetil (MMF) in three cases and post-transplant cyclophosphamide, tacrolimus and MMF in the four haploidentical transplant recipients.

Characteristics of the T-cell products

T-cell product characteristics are shown in Figure 1. Mean fold expansion of cell number in culture was 6.8-fold (range 2.6–22.4) and post-thaw viability mean 82% (range 70–99; Figure 1a–c). Products were mainly $CD3^+$ cells (mean 95%, range 89–99), $CD4^+$ and $CD8^+$ cells comprised a mean of 55% and 37% respectively. NK, monocyte and B cells means were 2.2%, 0.2% and 1.2% respectively; T-cells were mainly T_{em} (mean 75%) and T_{cm} (mean 20%; Figure 1d). Antigen specificity measured by MHC tetramer (available for six epitopes in four cases) comprised 28% of $CD8^+$ cells (range 2–79). The mean percentage of interferon (IFN)-γ producing $CD3^+$ cells (all antigens) measured by intracellular cytokine staining was 23% (range 8–60). The proportion of antigen-specific cells that reacted to individual antigens is shown in Figure 1e. CMV-responsive cells comprised a mean of 39% of reactive T-cells in seropositive donors. Responses to EBV antigens displayed the most variability (mean for all EBV antigens 4%, three cases of no EBV-specific

Table 1. Participant characteristics

Patient	Age, (years)	Sex	Transplant indication	Conditioning HSCT, HLA match	T-cell depletion (*in vivo*)	GVHD prophylaxis	CMV serostatus R/D	EBV serostatus (R/D)	VZV serostatus (R/D)	Day of T-cell infusion	Reason for delay in T-cell infusion[a]
1	56	F	NK leukaemia relapsed post-ASCT	RIC, MMSD[b]	ATG 30 mg	Cycl MMF	Pos/Pos	Pos/Pos	Pos/Pos	76	Active CMV, EBV, steroids, cardiomyopathy, skin GVHD
2	60	F	CLL complex karyotype	RIC, MUD	ATG 30 mg	Cycl MTX[c]	Pos/Pos	Pos/Pos	Pos/Pos	52	Active CMV
3	53	M	AML CR1	RIC, MSD	ATG 15 mg	Cycl MMF	Pos/Neg	Pos/Pos	Pos/Pos	31	–
4	45	M	AML CR1	MAC, MSD	–	Cycl MTX[d]	Neg/Neg	Pos/Pos	Pos/Pos	40	Severe mucositis, deconditioned, electrolyte abnormalities
5	50	M	AML CR1 complex cytogenetics	MAC, MSD	–	Cycl MTX[c]	Pos/Pos	Pos/Pos	Pos/Pos	37	Renal impairment due to Cycl toxicity
6	44	M	EBV + NK lymphoma/ HLH PR	RIC, haplo	–	PT Cy tacro MMF	Pos/Pos	Pos/Pos	Pos/Pos	28	–
7	66	F	AML CR2	RIC, haplo	–	PT Cy tacro MMF	Pos/Pos	Pos/Pos	Pos/Pos	38	Active CMV, BKV
8	26	M	ALL CR3	RIC, haplo[e]	–	PT Cy tacro MMF	Pos/Neg	Pos/Neg	Neg/Pos	59	Active CMV, skin GVHD
9	49	M	NHL mult relapse	RIC, haplo	–	PT Cy tacro MMF	Neg/Pos	Pos/Pos	Pos/Pos	28	–
10	60	F	AML CR1	RIC, MSD	–	Cycl MMF	Pos/Pos	Pos/Pos	Pos/Pos	28	–
11	36	M	AL biphenotypic CR1	MAC, MUD	ATG 20 mg	Cycl MTX[f]	Pos/Pos	Pos/Pos	Pos/Unk	32	–

ASCT, autologous stem cell transplant; ATG, antithymocyte globulin (rabbit, Fresenius); Cycl, cyclosporine; MMF, mycophenolate mofetil; MMSD, mismatched sibling donor; MSD, matched sibling donor; MTX, methotrexate; PR, partial remission; PT Cy, post-transplant cyclophosphamide; RIC, reduced intensity conditioning; tacro, tacrolimus; unk, unknown.

[a] Beyond day 35 post-transplant.

[b] HLA-B1 antigen mismatch.

[c] MTX 15 mg m^{-2} D1, 10 mg m^{-2} D3,6,11.

[d] D11 MTX omitted due to mucositis.

[e] 2nd allogeneic stem cell transplant.

[f] MTX 10 mg m^{-2} D1, 3, 6.

response in seropositive donors). VZV and influenza comprised 16% and 30% respectively. Adenovirus, Aspergillus and BKV responses made up a median of 8%, 4% and 2% respectively (Figure 1e). Of 10 products in which antigen-specific response testing was available, six responded to all possible pathogens, four had one pathogen to which there was no response (EBV $n = 3$ and VZV $n = 1$; Figure 1f).

Administration of T-cell product

Infusion day varied from day +28 to +76 post-transplant. Reasons for delay in T-cell infusion were active viral reaction with CMV, EBV and/or BKV ($n = 4$), severe mucositis and deconditioning ($n = 1$) and renal impairment ($n = 1$; Table 1). Two patients were receiving prednisone at doses under 0.5 mg kg^{-1} daily at the time of T-cell infusion, both for acute graft-versus-host disease (GVHD) of the skin. In both patients, the dose of prednisone was being weaned at the time of infusion.

Adverse events after administration of T-cell product

There were no acute infusion related adverse events. GVHD, relapse and death are shown in

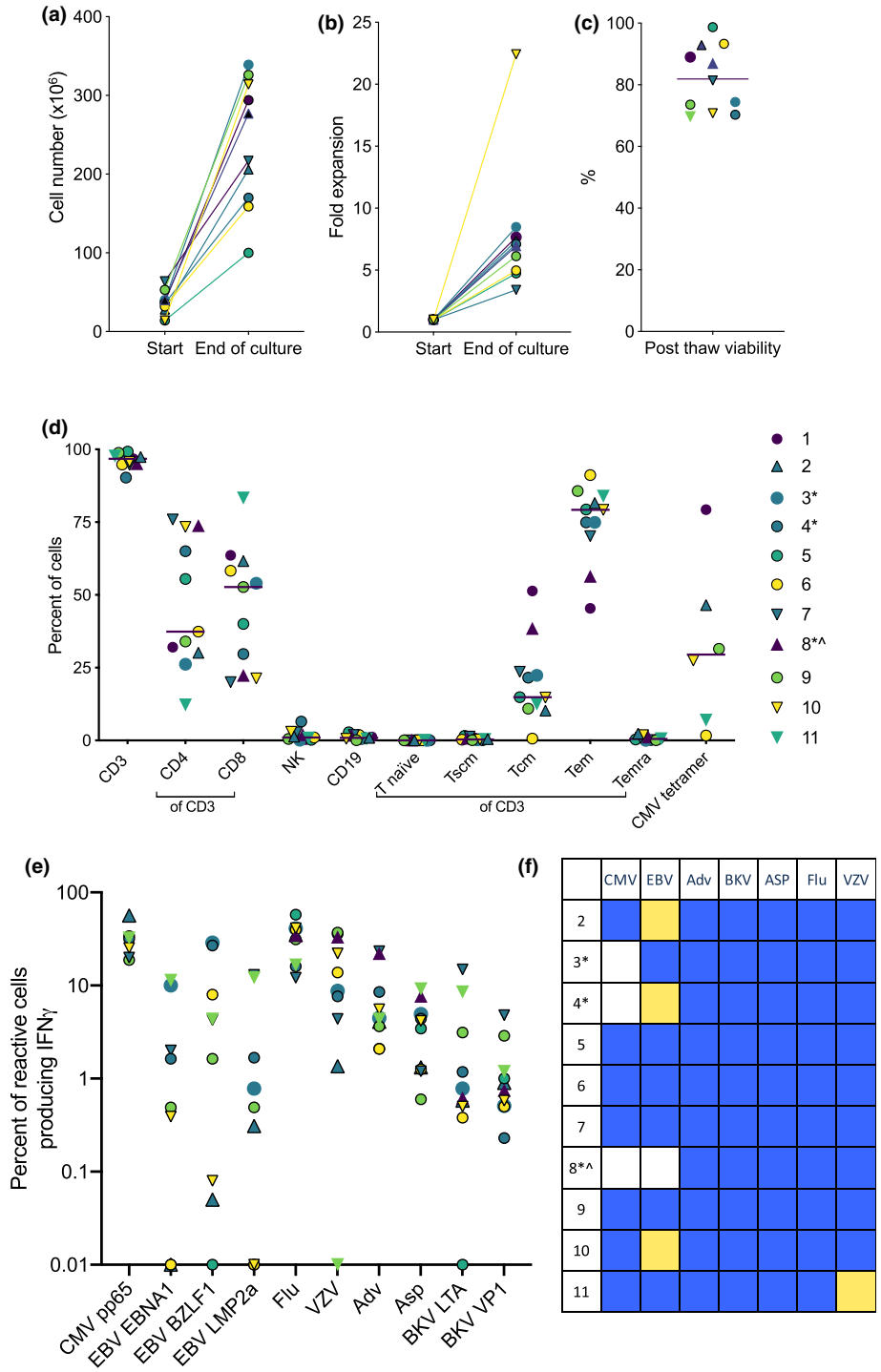

Figure 1. Characteristics of 11 multi-pathogen T-cell products at completion of expansion *in vitro*. **(a)** Total T-cell numbers in culture. **(b)** Fold expansion of cell number in culture. **(c)** Post-thaw viability as measured by flow cytometry. **(d)** Immunophenotype of final T-cell products (tetramer is % of $CD8^+$ cells; see Supplementary table S3 for epitopes and HLA restriction). **(e)** Proportion of each product's antigen-specific cells responding to each antigen (n = 10 products; values are after subtraction of negative control). **(f)** Responsiveness of individual products to each pathogen (n = 10 products). Blue blocks indicate response by cytokine flow cytometry for interferon gamma > 1%, yellow blocks < 1%, clear blocks donor seronegative for that pathogen; all values are after subtraction of negative control. Symbols refer to the same patient in all subfigures. * = donor CMV seronegative; ^ = donor EBV negative; Adv, adenovirus; ASP, aspergillus; BKV, BK virus; CMV, cytomegalovirus; EBV seronegative; EBV, Epstein–Barr virus; Flu, influenza; VZV, varicella zoster virus.

Table 2. Graft-versus-host disease, relapse and death

Patient	aGVHD	Grade aGVHD	Organs involved	2nd line therapy	Day post-transplant aGVHD	Day post-transplant T-cell infusion	Corticosteroid administration post-transplant (Y/N); day post-transplant started; max dose (mg kg^{-1})	cGVHD	cGVHD severity	Death, cause of death, day post-Tx
1	Y	II	Skin	N	26	76	Y; 26; 0.5	N	–	Dead, relapse D158[a]
2	N	–	–	–	–	52	N	N	–	Alive
3	N	–	–	–	–	31	Y; 35; 2	N	–	Alive[a]
4	Y	III	Skin/GI	N	70	40	Y; 81; 2	Y	Moderate	Alive
5	Y	IV	Skin/GI	N	68	37	Y; 75; 2	Y	Moderate	Dead, relapse D765
6	N	–	–	–	–	28	N	N	–	Dead, relapse D147[a]
7	Y	II	Skin	N	109	38	Y; 116; 1	N	–	Alive
8	Y	II	Skin	N	17	59	Y; 17; 0.5	N	–	Dead, relapse D280
9	Y	I	Skin	N	36	28	Y; 38; 0.5	N	–	Dead, multi-organ failure d138[a]
10	Y	III	GI	N	63	28	Y; 73; 2	N	–	Alive
11	Y	IV	Skin/GI	Y^	74	32	Y; 76; 2	Y	Mild	Dead, relapse d743

[a] Patient 1 censored day 115 for relapse, patient 6 censored d52 for relapse, patient 3 censored d81 after receiving 3rd party CMV-specific T-cell infusion, and patient 9 censored d66 after receiving 3rd party CMV-specific T-cell infusion.
^ Second line therapy with anti-lymphocyte globulin (ATGAM) and etanercept.

Table 2. In six cases, acute GVHD developed after T-cell infusion (median day post-transplant 71, range 36–109; median day post-infusion 33 days, range 8–71). In two additional cases (patient nos 1 and 8), acute skin GVHD developed prior to T-cell infusion but resolved sufficiently to allow T-cell infusion at a later date. Neither patient developed recurrent acute GVHD after T-cell infusion. Grades of acute GVHD in the six patients with acute GVHD after T-cell infusion were I (n = 1), II (n = 1), III (n = 2) and IV (n = 2). Grades I and II GVHD affected skin only while grade III/IV acute GVHD affected skin and gut in three cases and gut only in one case. All four patients with grade III/IV acute GVHD were at high risk of GVHD (omission of day 11 methotrexate (n = 2), early cessation of ciclosporin post-transplant due to renal impairment (n = 1) and use of reduced doses of ATG and post-transplant methotrexate (n = 1)). Three patients developed chronic GVHD by NIH consensus criteria, mild in one case and moderate in two cases.

Survival

After a median follow-up period of 390 days, 6 of the 11 patients recruited to the study died (day post-transplant 138–743; Table 2). Of these, five died from recurrence of the malignant disease for which their transplant was performed. One patient (patient no. 9) who underwent transplant after multiple courses of chemotherapy for non-Hodgkin lymphoma which was not in remission at the time of transplant died of multi-organ failure 138 days post-transplant.

Immune reconstitution

T-cell recovery

The majority of patients receiving multi-pathogen T-cells showed an increase in peripheral blood T-cell numbers within 30 days of infusion. This increase consisted predominantly of antigen-experienced activated effector memory $CD8^+$ T-cells expressing CD27, CD45RO, CD62L, CD38, CD57 and CD45RA (Figure 2a).

ELISPOT analysis

ELISPOT analysis was used to determine interferon-γ release from lymphocytes following stimulation with individual pathogens (Figure 2b). CMV responses were dominant and increased in all cases. For other pathogens, there was a small increase in IFN-γ response in the majority of cases, with occasional patients showing a substantial increase after infusion (EBV n = 1, BKV n = 1, VZV n = 1, aspergillus n = 1).

Effect of corticosteroid administration on lymphocyte number and phenotype

Nine patients commenced corticosteroid treatment after transplant, in all but one case for acute GVHD. Patient 9 developed a maculopapular rash on day 35 post-transplant, 7 days after T-cell infusion, which was treated with prednisone for presumed GVHD. Skin biopsy showed findings consistent with a drug rash and prednisone was rapidly weaned. Absolute lymphocyte counts fell from a mean of 0.9×10^9 L^{-1} (range 0.1–2.6) to 0.2 (range 0.0–0.6) at a mean of 11 days after commencing corticosteroids ($P = 0.035$; Supplementary figure 1). To determine the effect of corticosteroids on antigen-specific T-cells, a detailed analysis was performed of a representative case (patient 10;

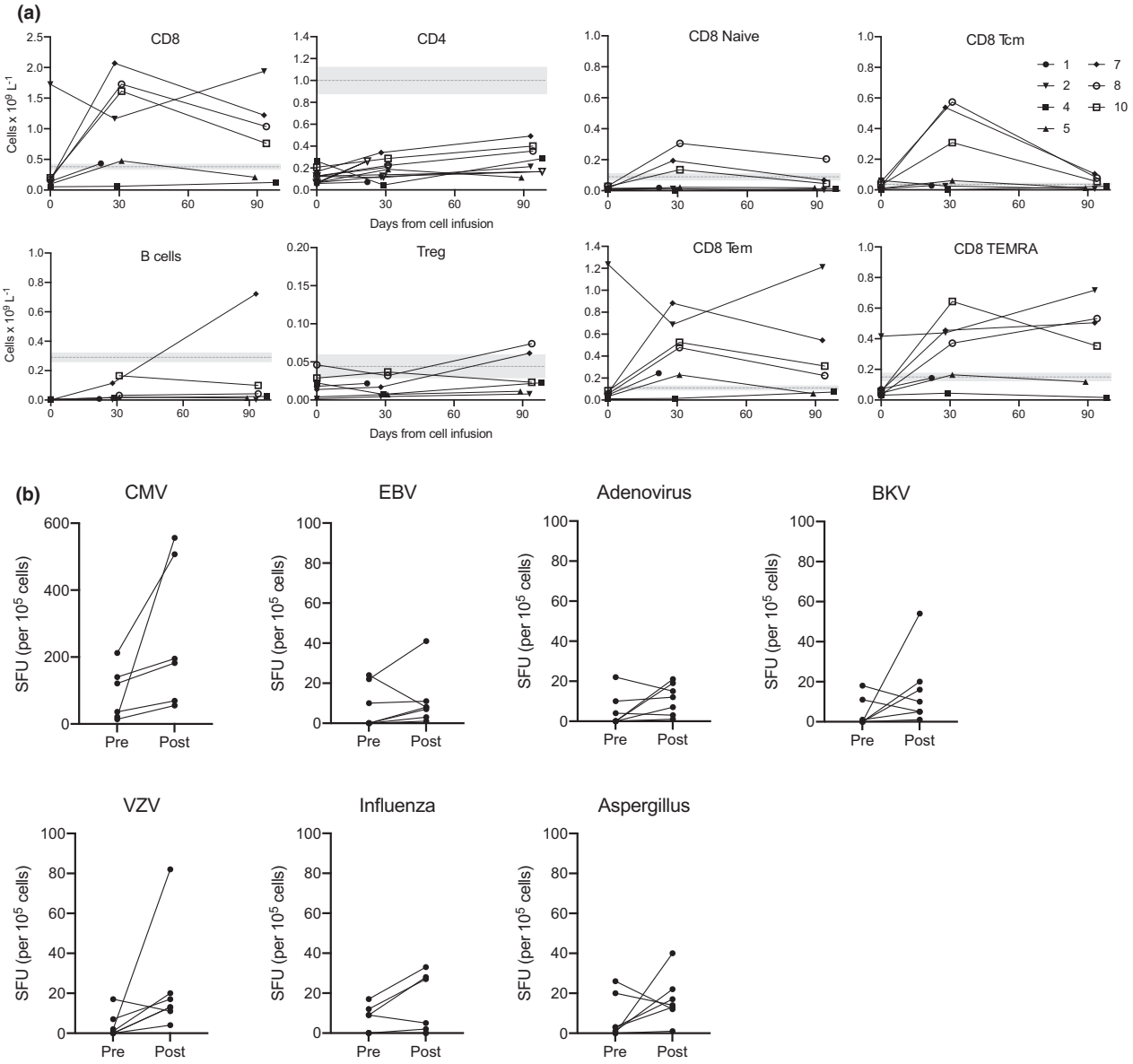

Figure 2. Immune reconstitution after T-cell infusion. **(a)** Recovery of peripheral blood T-cell subsets following infusion of multi-pathogen T-cell product as measured by mass cytometry ($n = 8$). Horizontal dashed line and shaded areas show mean and standard error in healthy individuals. **(b)** Pathogen-specific immune responsiveness measured by IFN-γ ELISPOT (pre-infusion vs peak response in the first 100 days post-infusion (spot-forming units (SFU) per 10^5 cells).

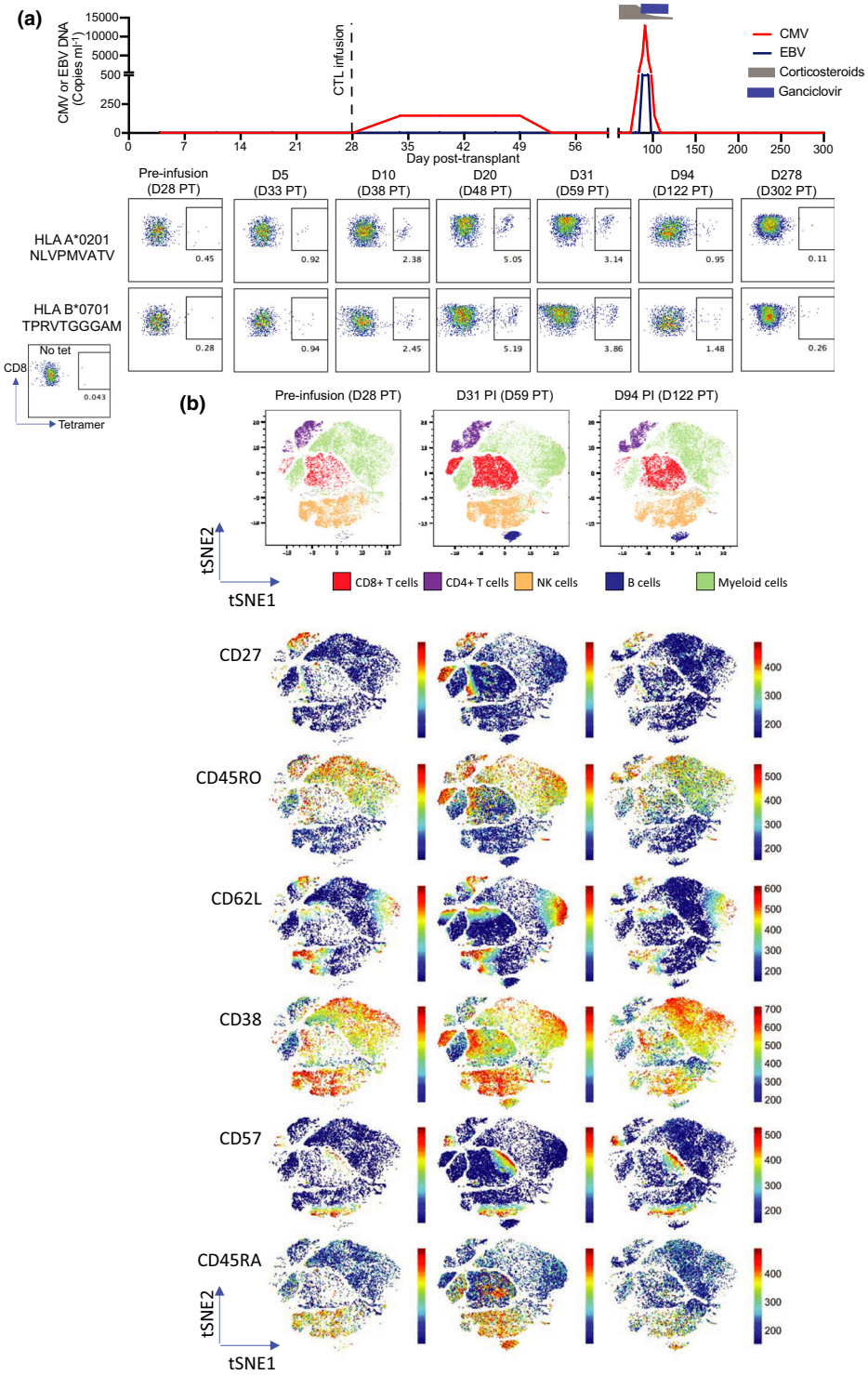

Figure 3. Effect of corticosteroids on immune reconstitution. **(a)** Time course of clinical events in patient 10 including staining with HLA-A*02:01 NLVPMVATV and HLA-B*07:01 TPRVTGGGAM tetramer in $CD8^+$ cells in peripheral blood pre- and post-T-cell infusion. **(b)** ViSNE of mass cytometry phenotype in peripheral blood in patient 10 pre-T-cell infusion, at day 31 post-infusion and at day 94 post-infusion. Upper panel shows major cell subsets. Increase, particularly in $CD8^+$ cell numbers, is shown at D31 post-infusion (intensity of red segment is proportional to cell number) with reduction at D93 post-infusion. Lower panel shows presence of the specific marker indicated within each subset. Cell-specific areas are as for upper panel but colour in each area refers to cell number on scale shown at right.

Table 3. CMV reactivation and treatment

Patient	CMV reactivation in relation to T-cell infusion	CMV reactivation free interval post-transplant (days)	Day of T-cell infusion	CMV at T-cell infusion (viral copies mL^{-1})	CMV reactivation free interval post-T-cell infusion (days)[a]	Corticosteroid administration post-transplant (Y/N); day post-transplant started; max dose (mg kg^{-1})	Peak CMV copy number pre; post-T-cell infusion (copies mL^{-1})	CMV disease (Y/N) (organ)	Antiviral drug[b] (Y/N); total number of days (pre/post-T-cell infusion)
1	Pre and post	3	76	0	4	Y; 26; 0.5	3970; < 150	N	Y; 20/0
2	Pre	17	52	0	–	N	8050; 0	N	Y; 12/4
3[c]	At and post	31	31	< 150	0	Y; 35; 2	< 150; 9170	Y (colitis)	Y; 0/29
4[c]	–	–	40	–	–	Y; 81; 2	0; 0	N	N
5	Pre and post	33	37	< 150	0	Y; 75; 2	< 150; 7520	Y (colitis)	Y; 0/38
6	Pre and post	3	28	14 300	0	N	14 300; 2140	N	N
7	At and post	38	38	< 150	0	Y; 116; 1	< 150; 249	N	N
8[c]	Pre and post	24	59	531	0	Y; 17; 0.5	14 600; 730	N	Y; 25/0
9	Post	38	28	0	10	Y; 38; 0.5	0; 124 000	Y (colitis)	Y; 21/2
10	Post	34	28	0	6	Y; 73; 2	0; 13 000	N	Y; 0/14
11	At and post	32	32	< 150	60	Y; 76; 2	< 150; 2700	N	Y; 0/22

–, CMV was not detected at infusion or subsequently.

[a]CMV reactivation free interval post-T-cell infusion was considered to be zero if CMV was detectable in the blood on the day of the T-cell infusion and on the following test.

[b]Ganciclovir or foscarnet at full therapeutic dose (adjusted for renal function).

[c]CMV seronegative donor (patient no. 4 also seronegative recipient).

Figure 3a). This patient developed low level CMV viraemia at day 34 post-transplant, 6 days after multi-pathogen T-cell infusion. This was followed by expansion of NLVPMVATV (HLA A*0201) and TPRVTGGGAM (HLA B*0701) tetramer positive populations that constituted over 10% of $CD8^+$ cells within 30 days of T-cell infusion leading to control of viral reactivation without antiviral therapy. After corticosteroids were administered for GVHD at post-transplant day 73 (D38 post-T-cell infusion), there was regression of total $CD4^+$ and $CD8^+$ cells, especially $CD8^+$ T-cells expressing CD27, CD45RO, CD62L, CD38, CD57 and CD45RA and tetramer positive $CD8^+$ T-cells (Figure 3b).

Viral reactivation

CMV

Eight of ten patients had their initial reactivation of CMV prior to or on the day of T-cell infusion (Table 3, Figure 4). In six patients (nos 3, 5, 7, 9, 10 and 11), peak post-transplant CMV copy number was observed after T-cell infusion (Figure 4b). All five patients with a maximum viral titre above 1000 IU mL^{-1} reactivated CMV after commencement of corticosteroids given for suspected or proven graft-versus-host disease (GVHD). Three patients (nos 3, 5 and 9) developed CMV colitis; the interval between T-cell infusion and steroid initiation in these patients was 4, 38 and 10 days and the patients received maximal steroid doses of 2, 2 and 0.75 mg per kg per day prednisone respectively. Patient 3 had received a T-cell product from a CMV seronegative donor and the product had no CMV specificity. He was eventually successfully salvaged with third-party CMV-specific T-cells on another trial.

EBV

EBV viraemia occurred in seven patients (Table 4). In six patients (patient nos 3, 4, 5, 6, 10 and 11) maximum EBV copy number occurred after T-cell infusion (Figure 5). Patient 6 underwent transplant for EBV associated lymphoma. Although EBV viraemia disappeared post-transplant and T-cell infusion, it subsequently recurred and was found to be associated with disease relapse. The patient was censored from the trial at the time of relapse. In the remaining five cases, EBV viraemia developed after commencement of corticosteroids. EBV viraemia exceeded 5000 copies per mL in only one case and none of the patients received treatment for viraemia. One patient (no. 1) was treated with

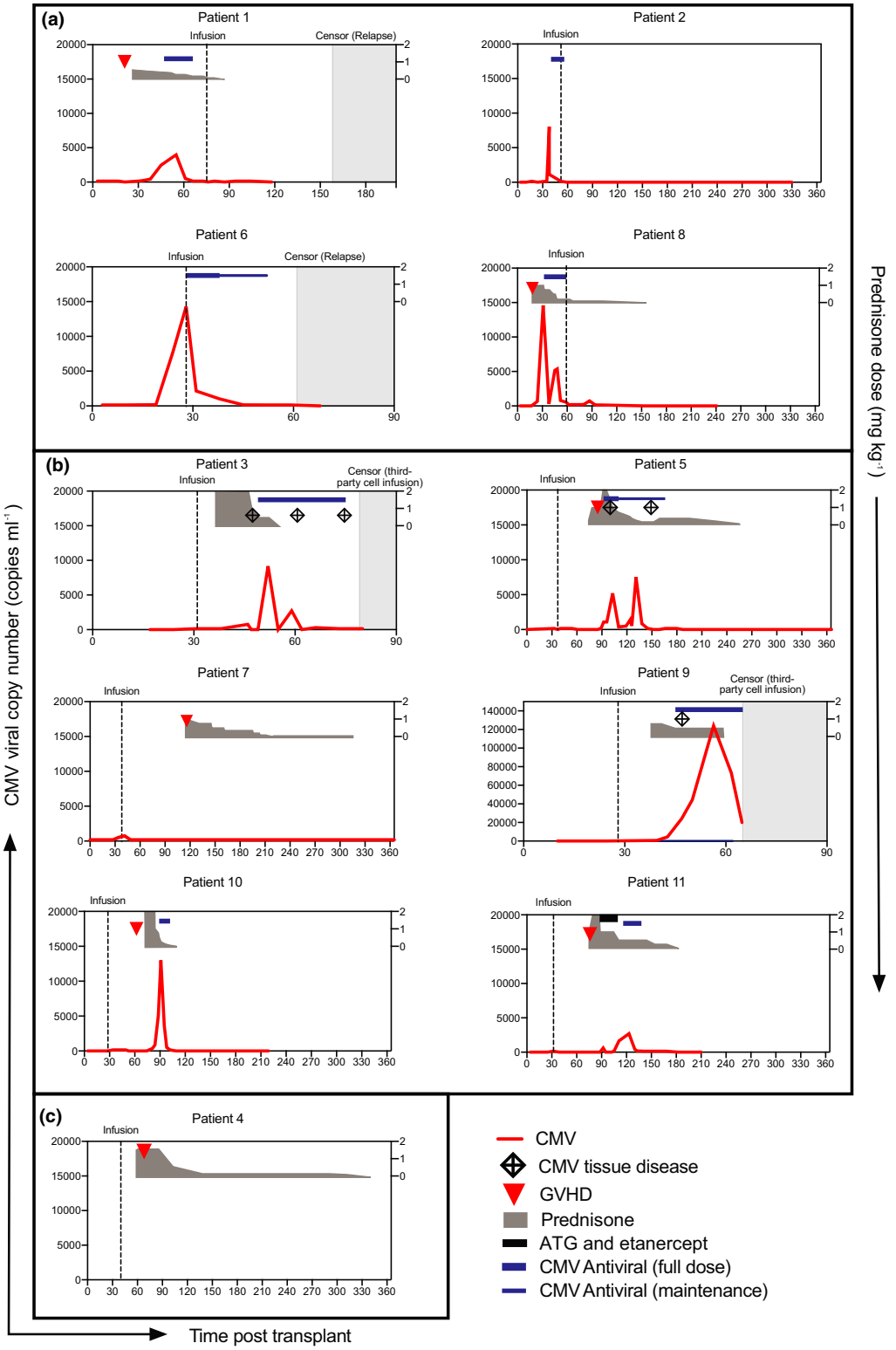

Figure 4. CMV infection. **(a)** Patients 1, 2, 6 and 8 with maximum CMV copy number prior to or at the time of T-cell infusion. **(b)** Patients 3, 5, 7, 9, 10 and 11 with maximum copy number after T-cell infusion. **(c)** Patient 4 with no CMV reactivation at any time. *x*-axis days post-transplant, left *y*-axis CMV DNA copies mL^{-1}, right *y*-axis corticosteroid dose (equivalent to prednisone dose in mg kg^{-1}; grey blocks). The vertical dashed line shows T-cell infusion. Shading indicates censor due to study withdrawal or relapse of original disease.

Table 4. EBV reactivation and treatment

Patient	EBV reactivation	Timing of EBV reactivation in relation to T-cell infusion	EBV reactivation free interval post-transplant (days)	Day of T-cell infusion	EBV at T-cell infusion (copies mL^{-1})	EBV reactivation free interval post-T-cell infusion (days)[a]	Corticosteroid administration post-transplant (Y/N); day post-transplant started; max dose (mg kg^{-1})	Peak EBV copy number pre; post-T-cell infusion (copies mL^{-1})	EBV disease (Y/N)	Antiviral drug[b] (Y/N)
1	Y	Pre and post	45	76	1000	0	Y; 26; 0.5	228 000; 1000	N	Y; ritux x 2 doses
2	N	–	–	52	–	–	N	–	N	N
3	Y	Post	59	31	–	28	Y; 35; 2	0; 1890	N	N
4	Y	Post	103	40	–	63	Y; 81; 2	0; 500	N	N
5	Y	Post	89	37	–	62	Y; 75; 2	0; 20 600	N	N
6	Y	Pre and post	3	28	1491	0	N	1194; 4720	N	N
7	N	–	–	38	–	–	Y; 116; 1	–	N	N
8	N	–	–	59	–	–	Y; 17; 0.5	–	N	N
9	N	–	–	28	–	–	Y; 38; 0.5	–	N	N
10	Y	Post	88	28	–	60	Y; 73; 2	0; 500	N	N
11	Y	Post	102	32	–	70	Y; 76; 2	0; 500	N	N

EBV was not detected at infusion or subsequently.

[a]EBV reactivation free interval post-T-cell infusion was considered to be zero if EBV was detectable in the blood on the day of the T-cell infusion and on the following test.

[b]Rituximab or chemotherapy.

two doses of rituximab, the first given prior to and the second after T-cell infusion. No further therapy was given. No cases of PTLD occurred.

Adenovirus

None of the 11 patients had adenovirus detected in blood or tissues at any time following transplant.

Influenza

Two patients (patient nos 5 and 6) had influenza A detected on nasopharyngeal swabs, one prior to and one 37 days after T-cell infusion. The former was treated with oseltamivir orally for 10 days with symptom resolution, the latter was not treated and symptoms resolved.

Varicella zoster virus

There were no cases of herpes zoster, and varicella zoster was not detected by laboratory testing in any patient.

BK virus

BK viruria was detected in 10 of 11 patients (Table 5, Figure 6) most commonly prior to or at the time of T-cell infusion (Figure 6a). BK viruria was controlled rapidly after T-cell infusion in all patients. In four patients BK virus developed only after T-cell infusion, in all cases in association with corticosteroid and/or other GVHD treatment (Figure 6b). BK virus infection was short lived in all cases although one patient (patient no. 4) developed brief symptomatic grade 2 haemorrhagic cystitis.

Aspergillus

Four patients had positive peripheral blood Aspergillus PCR post-transplant. In two cases positive tests occurred prior to T-cell infusion. Two patients had a single positive peripheral blood Aspergillus PCR after T-cell infusion but neither of them nor any other patient required systemic antifungal treatment for a proven or probable invasive fungal infection post-transplant.

Clone tracking

To determine the fate of individual T-cell clones, TCR sequences identified in patient 10 prior to infusion and in the infused T-cell product were tracked up to day 278 post-infusion. Public CMV-specific clonotypes (cross referenced with the VDJ database vdjdb.CDR3.net) expanded in the patient

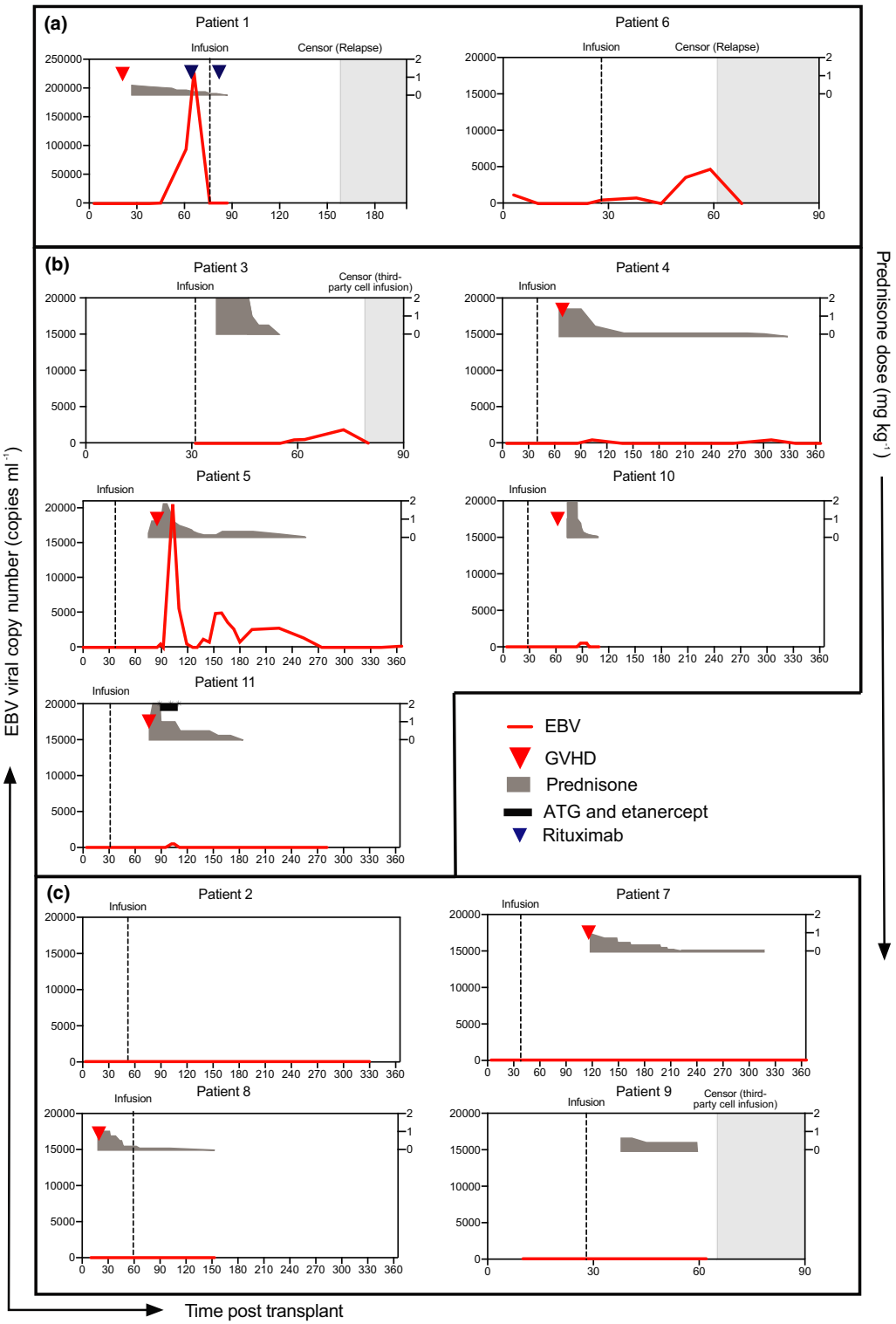

Figure 5. EBV infection. **(a)** Patients 1 and 6 with EBV detected prior to or at the time of T-cell infusion. Patient 6 had EBV viraemia associated with relapse of EBV driven lymphoma. **(b)** Patients 3, 4, 5, 10 and 11 with EBV detected after T-cell infusion. **(c)** Patients 2, 7, 8 and 9 with no EBV reactivation at any time. *x*-axis days post-transplant, left *y*-axis EBV DNA copies mL^{-1}, right *y*-axis corticosteroid dose (equivalent to prednisone dose in mg kg^{-1}; grey blocks). The vertical dashed line shows T-cell infusion. Shading indicates censor due to study withdrawal or relapse of original disease. The downward blue arrow indicates rituximab administration.

Table 5. BKV reactivation

Patient	BKV reactivation	Timing of BKV reactivation in relation to T-cell infusion	BKV reactivation free interval post-transplant (days)	Day of T-cell infusion	BKV at T-cell infusion (copies mL^{-1})	BKV reactivation free interval post-T-cell infusion (days)[a]	Corticosteroid administration post-transplant (Y/N); day post-transplant started; max dose (mg kg^{-1})	Peak BKV copy number pre; post-T-cell infusion (copies mL^{-1})	Days to maximum copy number post-T-cells	BKV disease symptomatic
1	Y	Pre, at and post	21	76	1.5×10^9	0	Y; 26; 0.5	1.5×10^9; 1.2×10^8	4	N
2	Y	Pre, at and post	38	52	9.9×10^9	0	N	9.9×10^9; 3.4×10^{10}	7	N
3	Y~	Pre, at and post	24	31	2.9×10^9	21	Y; 35; 2	2.7×10^{10}; 8.4×10^9	21	N
4	Y~	Post	86	40	NT	46	Y; 81; 2	0; 7×10^9	63	Y
5	N~	–	–	37	–	–	Y; 75; 2	–	–	N
6	Y~	At and post	28	28	$> 10 \times 10^{10}$	0	N	$> 10 \times 10^{10}$; 5.8×10^9	17	N
7	Y~	Pre, at and post	31	38	1.6×10^{10}	0	Y; 116; 1	8.7×10^{10}; 3.1×10^{10}	14	N
8	Y	Pre and post	52	59	NT	3	Y; 17; 0.5	1.9×10^8; 1.7×10^{10}	3	N
9	N	–	–	28	–	–	Y; 38; 0.5	–	–	N
10	Y	Post	34	28	NT	6	Y; 73; 2	0; 4.6×10^9	81	N
11	N	–	–	32	–	–	Y; 76; 2	–	–	N

NT, not tested; –, BKV was not detected at infusion or subsequently; ~, BK virus detected in blood on at least 1 occasion.
[a]BKV reactivation free interval was considered to be zero if BKV was detectable in the urine on the day of the T-cell infusion and on the following test.

by day 30 post-infusion during CMV viraemia and contracted thereafter (Supplementary figure 2a). Public EBV-specific clonotype proportions were smaller than CMV in all samples but also expanded maximally at the day 30 time point in the absence of detectable EBV viraemia (Supplementary figure 2a). In both cases, the dominant clones were present in both the infusion product and the pre-infusion sample. TCR clones shared with the day 278 sample were plotted to assess when the long-term clones became established in patient 10. The day 94 and day 278 had a larger number of shared clones than other samples and the Morisita similarity index (comparing each time point with the final one on D278) rose over time indicating that in this patient the long-term clonality was largely established by day 94 (Supplementary figure 2b and c).

DISCUSSION

We manufactured and prophylactically administered donor-derived T-cells specific for seven infectious pathogens that cause clinical disease in allogeneic stem cell transplant recipients. Generation of the multi-pathogen T-cell product was feasible and there were no acute infusion related toxicities. We observed acute GVHD including moderate and severe grades in patients that received T-cell infusions and we cannot exclude the possibility that the T-cell infusions contributed to the development of acute GVHD in these patients. However GVHD is a risk in all allogeneic stem cell transplant recipients and in this study we administered antigen-specific T-cells at a median of 37 days post-transplant when the risk of GVHD is greatest. Our population was also at relatively high risk. Only 4 of the patients had matched sibling donors, the remainder receiving transplants from unrelated or HLA mismatched donors. No patient received a graft that had been *in vitro* T-cell depleted. All four patients with grades III/IV disease had additional risk factors including omission of day 11 methotrexate ($n = 2$), early cessation of ciclosporin post-transplant due to renal impairment ($n = 1$) and use of reduced doses of ATG and post-transplant methotrexate ($n = 1$) that occurred after recruitment to the study. Across the three years during which patients were recruited to the study, the overall rate of acute

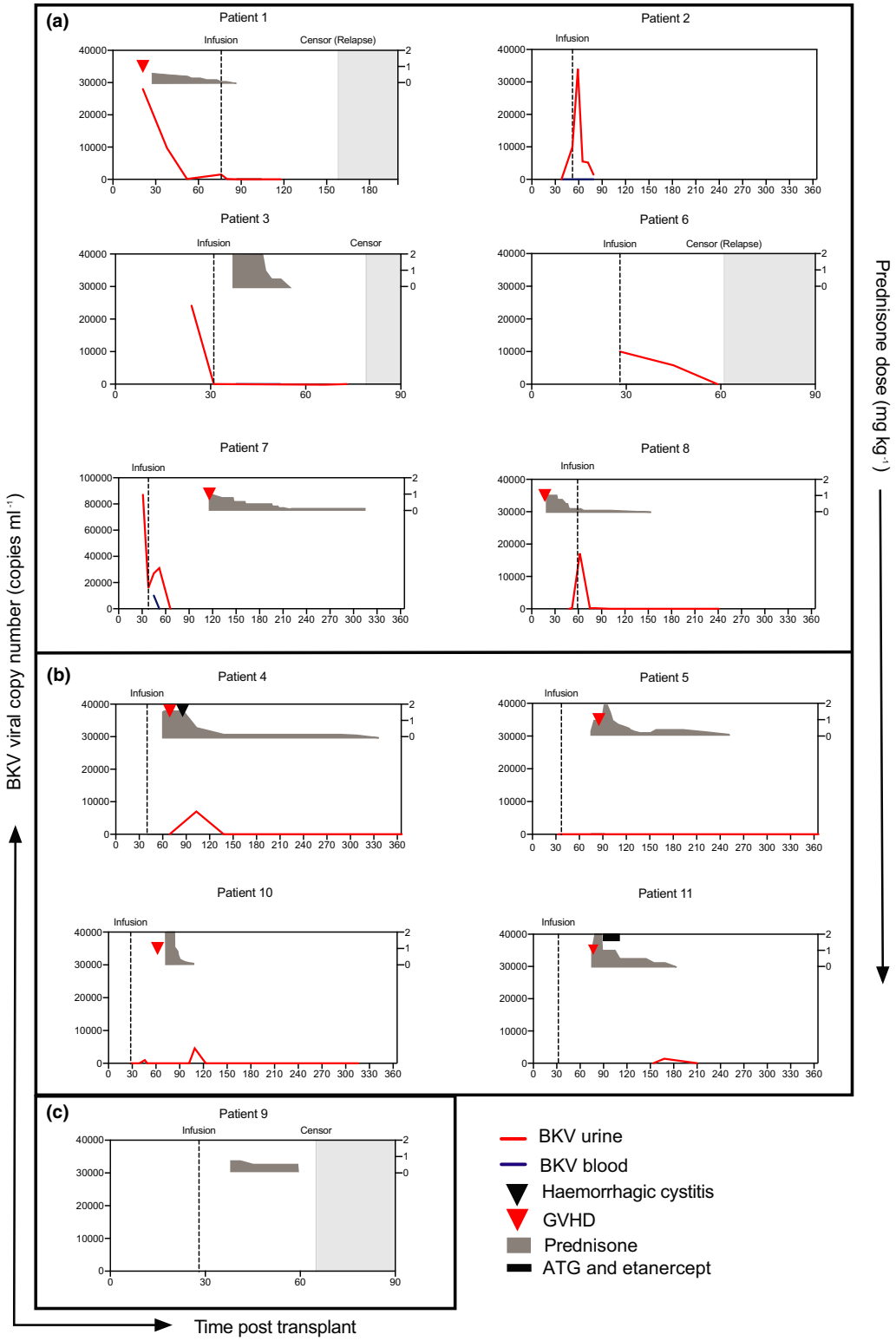

Figure 6. BKV infection. **(a)** Patients 1, 2, 3, 6, 7 and 8 with BKV in urine prior to, at and after T-cell infusion. **(b)** Patients 4, 5, 10 and 11 with BKV in urine at and after T-cell infusion. **(c)** Patient 9 without BKV in urine at any time. *x*-axis days post-transplant, left *x*-axis copies BKV DNA copies mL^{-1} in urine (red) and blood (blue), right *y*-axis corticosteroid dose (equivalent to prednisone dose in mg kg^{-1}; grey blocks). Shading indicates censor due to study withdrawal or relapse of original disease. Inverted diamond shows episode of haemorrhagic cystitis.

GVHD at our centre was 35% with 18% suffering grade III/IV disease. The confidence interval of GVHD incidence is wide given the small numbers in this study and it is therefore conceivable that the GVHD we observed was consistent with our previous experience and the risk factors involved.[24,25] Of note, previous studies of donor-derived pathogen-specific T-cells that showed low GVHD rates recruited patients receiving transplant regimens associated with significantly lower rates of GVHD[26,27] and enrolled patients in whom GVHD had not developed after transplant thus selecting a particularly low risk cohort. In contrast, our patients were enrolled prior to transplant and received conditioning regimens without $CD34^+$ cell selection, constituting a more typical group of transplant recipients.

Only one previous study has targeted fungi with adoptive T-cell immunotherapy and it did not administer fungal T-cells prophylactically.[19] Previously, T-cells recognising five viral pathogens (CMV, EBV, adenovirus, HHV6 and BK virus) have been simultaneously targeted by adoptive immunotherapy.[11] Our data extend the range of viral pathogens targeted and add fungal infections as a target for adoptive immunotherapeutic approaches. Despite the presence of GVHD and the use of intermediate to high dose corticosteroids, we observed only 1 non-relapse death in the 11 patients reported here. A twelfth patient whose information was not included due to refusal to attend for regular follow-up also remains alive. No patient died of infection. However, we observed three cases of CMV colitis, all following administration of high doses of corticosteroids after T-cell infusion; one of these had a CMV seronegative donor from whom no CMV-specific T-cells were expanded. This patient was salvaged with third-party CMV-specific T-cells on another trial. A notable feature of the three patients who developed CMV colitis was the short interval between T-cell infusion and corticosteroid administration. Three other patients who received similar doses of corticosteroids but starting later after T-cell infusion (including one who received second line therapy for GVHD) required no or relatively brief anti-CMV treatment. In five patients EBV was detected only after T-cell infusion, a finding that may represent shedding from cells destroyed as part of an immune attack on EBV infected tissues.[28] In all five patients (including one patient who reactivated EBV while receiving corticosteroids, ATG and etanercept), EBV resolved without specific treatment. Similarly, although 10 of the 11 patients had BK virus identified in urine after transplant, there was only one brief episode of clinical haemorrhagic cystitis despite high viral copy numbers in some patients. Finally despite the use of intermediate to high dose corticosteroids and second line aGVHD treatment, no patient developed a systemic fungal infection or required escalation of standard antifungal prophylaxis.

Administration of the T-cell product was followed by an increase in peripheral blood T-cell numbers 30 days after infusion. The increase comprised principally activated antigen-experienced $CD8^+$ T-cells. Detailed analysis of one patient using CyTOF showed rapid reconstitution of CMV immunity but clearly demonstrated the susceptibility of these cells to corticosteroids. There was little change in the number of $CD4^+$ T-cells, regulatory T-cells or B cells after T-cell infusion. Our data do not allow us to identify the origin of the expanded $CD8^+$ T-cells as the stem cell donor was the source of both the original stem cell infusion and the T-cell product. However in one patient, the most common CMV and EBV public clones at day 30 after infusion were also the most common in the T-cell product. Nine months after infusion, these clones had substantially contracted. T-cell infusion was followed by evidence of improved functional immunity only to CMV. We previously observed that prophylactic infusions of antigen-specific T-cells do not induce increases in detectable peripheral blood immunity in the absence of antigenemia.[9] Consistent with this observation, enhanced CMV ELISPOT responses were numerically greatest in those who developed CMV viraemia. Only sporadic improvement in immune reconstitution to other infections was observed.

Our study has several limitations, and the data should be interpreted with caution. Most importantly, only a small number of patients with different diagnoses and undergoing various forms of transplant received the cellular therapy in this early phase trial. There was no comparator cohort, rendering it very difficult to draw conclusions about efficacy and potential adverse effects. Nevertheless, our data support the feasibility of *ex vivo* manufacture of T-cells targeting multiple opportunistic pathogens although they sound a note of caution regarding the risk of graft versus host associated with infusion of cells targeting multiple infectious antigens soon after allogeneic

stem cell transplant. Further prophylactic use of antigen-specific T-cell immunotherapy should only be combined with GVHD prevention methods such as $CD34^+$ selection or other strategies such as TCR$\alpha\beta$ T-cell, alloreactive or naïve T-cell depletion and should be cautious in the cell numbers used, the range of pathogens targeted and the need to monitor for GVHD risk.[29–31] We recently commenced a pilot trial in which transplantation of *in vitro* isolated $CD34^+$ stem cells will be followed by administration of limited numbers of narrowly targeted infection- and tumor-specific T-cells (ACTRN12618001090202) and have seen no GVHD in the first two patients treated using this approach. If similar results are replicated during pilot trial recruitment, we will move to establishment of a formal phase 1/2 study examining the safety and efficacy of this approach in allogeneic transplantation.

METHODS

Study design and participants

The study was conducted as a single-arm, open label phase I trial. Allogeneic HSCT recipients and their donors were recruited prior to transplant. Detailed inclusion and exclusion criteria are provided in Supplementary table 1. Written informed consent was obtained in accordance with the Declaration of Helsinki. The study was approved by the Western Sydney Local Health District Human Research Ethics Committee. This study was registered on the Australian and New Zealand Clinical Trial Registry as trial NCT02843321.

Generation of multi-pathogen T-cell product

The granulocyte colony-stimulating factor (G-CSF) primed apheresis product from each patient's stem cell donor was used as the starting material for *ex vivo* T-cell expansion as previously described.[9,32] Monocyte derived dendritic cells (moDCs) were pulsed with overlapping MACS GMP PepTivators for HCMV pp65, AdV5 Hexon, EBV BZLF1/LMP2A/EBNA-1 (Miltenyi Biotech, Bergisch Gladbach, Germany), peptide pools (15mers overlapping by 11 peptides) for BKV proteins LTA and VP1, or with varicella zoster vaccine, influenza vaccine (CSL, Melbourne, VIC, Australia) or lysate of *Aspergillus fumigatus* (Miltenyi Biotech). Irradiated pulsed moDCs were co-cultured with the monocyte-depleted fraction of G-CSF primed apheresis products isolated by Ficoll-Paque (GE Healthcare, Chicago, IL, USA) gradient centrifugation. After 7 days, cultures from individually stimulated products were combined and restimulated with peptide/lysate-pulsed moDCs. Cultures were continued for up to 21 days, with the addition of 20 U mL^{-1} interleukin-2, increasing to 50 U mL^{-1} from day 14 to 21. T-cell products were cryopreserved for later administration.

Assessment of T-cell product

Cell dose was based on post-thaw viability. Standard pathogen-specific T-cell product release criteria were applied as described previously[9] and in Supplementary table 2. Flow cytometry for product release was performed using monoclonal antibodies directed against CD3, CD4, CD8, CD14, CD19 and CD56 (BD Biosciences, San Jose, CA, USA). Viability was assessed using 7-amino-actinomycin D (BD Biosciences) or hydroxystilbamidine (Life Technologies, Carlsbad, CA, USA). Viral antigen specificity and HLA-restricted epitope recognition were assessed using phycoerythrin-conjugated virus-specific iTAgMHCclass I human tetramers (Beckman Coulter, Brea, CA, USA; epitopes and HLA restriction are shown in Supplementary table 3) and by intracellular cytokine flow cytometry (ICFC) after T-cell stimulation as previously described[33] using the same antigens used for product manufacture (a representative example of ICFC is shown in Supplementary figure 3). T-cell subsets were assessed using monoclonal antibodies directed against CD3, CD4, CD8, CD95, CD45RA, CD62L and Zombie (BD Biosciences) and defined as naïve $CD45RA^+CD62L^+$, stem cell memory (T_{scm}) $CD45RA^+CD62L^+CD95^+$, terminally differentiated effector (T_{emra}) $CD45RA^+CD62L^-$, effector memory (T_{em}) $CD45RA^-CD62L^-$ and central memory (T_{cm}) $CD45RA^-CD62L^+$. Flow cytometry data were acquired on a FACSCanto II or LSRFortessa (BD Biosciences) and analysed with FlowJo software (version 10.0.8r1; Tree Star, Inc., Ashland, OR, USA).

Participant treatment and monitoring

Recipients were treated with a single infusion of 2×10^7 T-cells per square metre of body surface area on or after day 28 post-transplant as infection prophylaxis. Antiviral and antifungal monitoring included weekly CMV and EBV monitoring by PCR in all cases. Testing for other infections was performed at the discretion of treating clinicians based on patient symptoms. Initiation of treatment, choice of anti-infective agent, dose and duration of therapy were according to standard institutional practice. Patients were monitored for 12 months from the final infusion of T-cells for evidence of infections, toxicity, and immune cell recovery.

Outcomes

The primary end point of the trial was safety of the T-cell infusion. Secondary endpoints included post-infusion immune reconstitution, incidence of targeted infections and incidence of acute and chronic GVHD. All adverse events were graded according to the National Cancer Institute's common terminology criteria for adverse events, version 4.03. Viral copy number was measured by quantitative PCR using the Roche-Cobas system (Hoffman La Roche, Germany). GVHD was graded according to standard criteria for acute and chronic GVHD.[34,35]

Immunological monitoring

Enzyme-linked immunospot assay (ELISPOT) was performed on post-infusion peripheral blood samples as previously

described[23] using the same antigens as manufacturing and ICS assays. Mass cytometry was used to describe global immune recovery in some patients pre-infusion, day 30 and day 90 post-infusion. A panel of 2 fluorescent-tagged and 38 metal-tagged monoclonal antibodies was used (antibodies and corresponding metal tag in Supplementary table 4). All pre-conjugated antibodies were purchased from Fluidigm, Toronto, Canada. All other antibodies were purchased in a carrier-protein-free format and conjugated with the metal isotope using the MaxPAR antibody conjugation kit (Fluidigm) according to the recommended protocol. Cells were acquired at a rate of 200–400 cells per second using a CyTOF 2 Helios upgraded mass cytometer (Fluidigm). Populations were gated manually and number of cells $\times 10^9$ L^{-1} calculated using lymphocyte and monocyte counts generated by the full blood count analyser on the day of sample collection (dual platform) as previously described.[33]

TCR sequencing

TCR sequence acquisition was performed as previously described.[36] RNA was isolated using Qiagen RNeasy Mini kits according to the manufacturer's instructions. (Quantitect RT, QIAgen, Chadstone, VIC, Australia). Using 5 μL of cDNA template per reaction, TCRβ transcripts were PCR amplified from the *ex vivo* expanded T-cell product and post-infusion sorted PBMC samples using high-fidelity Q5 polymerase (NEB) and a mix of 19 Trbv-specific forward primers and a single Trbc-specific reverse primer. Forward and reverse primers had distinct 5′ overhang adapter sequences that enabled addition of sample-specific indices and P5/P7 sequencing adapters in a second PCR using the Illumina Nextera XT DNA library preparation kit. Before the second PCR, magnetic beads (Agencourt AMPure XP, Beckman Coulter) were used to enrich amplicons > 100 bp. Equimolar amounts of amplicons were pooled into a single tube, concentrated using magnetic beads and 300–500 bp amplicons were gel-purified before sequencing on an Illumina NextSeq machine, with a short read 1 of 6 bases followed by a read 2 of 145 bases. For TCR sequence processing, see the Supplementary Methods.

Statistical analysis

Demographics, clinical characteristics, viral reactivation, transplantation outcomes and immunological observations were tabulated for presentation or visualised with Prism v8.4.2 for Mac (GraphPad Software Inc., La Jolla, CA, USA). The paired *t*-test was used to compare means. Mass cytometry analysis was analysed using FlowJo X 10.0.7r2 software (FlowJo, LLC, Ashland). The visualised *t*-distributed stochastic neighbourhood embedding (ViSNE) algorithm (implemented in FlowJo as a plugin) was utilised to perform dimensionality reduction and visualisation of immune subsets across samples.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

David Gottlieb: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing. **Leighton Clancy:** Conceptualization; Formal analysis; Methodology; Supervision; Writing-review & editing. **Barbara P Withers:** Investigation; Methodology; Visualization; Writing-review & editing. **Helen McGuire:** Investigation; Supervision; Visualization; Writing-review & editing. **Fabio Luciani:** Formal analysis; Software; Visualization; Writing-review & editing. **Mandeep Singh:** Methodology; Software; Writing-review & editing. **Brendan Hughes:** Software; Writing-review & editing. **Brian Gloss:** Formal analysis; Validation; Visualization. **David Kliman:** Investigation; Project administration; Writing-review & editing. **Chun-Kei-Kris Ma:** Methodology; Project administration; Writing-review & editing. **Shyam Panicker:** Investigation; Writing-review & editing. **David Bishop:** Investigation; Writing-review & editing. **Ming-Celine Dubosq:** Investigation; Writing-review & editing. **Ziduo Li:** Data curation; Formal analysis; Visualization. **Selmir Avdic:** Data curation; Formal analysis. **Kenneth Micklethwaite:** Conceptualization; Investigation; Methodology; Project administration; Writing-review & editing. **Emily Blyth:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.