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Transplant-induced reactivation of murine cytomegalovirus immediate early gene expression is associated with recruitment of NF-κB and AP-1 to the major immediate early promoter

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Reactivation of latent human cytomegalovirus is a significant infectious complication of organ transplantation and current therapies target viral replication once reactivation of latent virus has already occurred. The specific molecular pathways that activate viral gene expression in response to transplantation are not well understood. Our studies aim to identify these factors, with the goal of developing novel therapies that prevent transcriptional reactivation in transplant recipients. Murine cytomegalovirus (MCMV) is a valuable model for studying latency and reactivation of CMV *in vivo*. We previously demonstrated that transplantation of MCMV-latently infected kidneys into allogeneic recipients induces reactivation of immediate early (IE) gene expression and epigenetic reprogramming of the major immediate early promoter (MIEP) within 48 h. We hypothesize that these events are mediated by activation of signalling pathways that lead to binding of transcription factors to the MIEP, including AP-1 and NF-κB. Here we show that transplantation induces rapid activation of several members of the AP-1 and NF-κB transcription factor family and we demonstrate that canonical NF-κB (p65/p50), the junD component of AP-1, and nucleosome remodelling complexes are recruited to the MIEP following transplantation. Proteomic analysis of recipient plasma and transcriptome analysis of kidney RNA identified five extracellular ligands, including TNF, IL-1β, IL-18, CD40L and IL-6, and three intracellular signalling pathways associated with reactivation of IE gene expression. Identification of the factors that mediate activation of these signalling pathways may eventually lead to new therapies to prevent reactivation of CMV and its sequelae.

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INTRODUCTION

Human cytomegalovirus (HCMV) is an opportunistic herpesvirus that has the ability to establish a lifelong latent infection and to reactivate. Reactivation of HCMV in transplant recipients is associated with CMV disease, increased risk of acute and chronic allograft rejection, infection with other opportunistic pathogens, graft failure and death (Razonable *et al.*, 2013). Due to the high prevalence of latent infection, more than 75 % of solid organ transplant recipients experience reactivation of latent HCMV (Fishman *et al.*, 2007). Although effective antiviral drugs have reduced the incidence of CMV-related post-transplant complications, use of these drugs is limited by their toxicity and the emergence of resistant strains (Razonable *et al.*, 2013). Thus, a greater understanding of the molecular events controlling reactivation from latency is needed to develop alternative strategies to prevent CMV disease.

Reactivation of HCMV has been associated clinically with conditions that generate an inflammatory milieu, including allograft rejection, sepsis and acute illness (Cook *et al.*, 1998;

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One supplementary figure is available with the online Supplementary Material.

Döcke *et al.*, 1994; Fietze *et al.*, 1994; Grattan *et al.*, 1989; Heininger *et al.*, 2001; Hibberd *et al.*, 1992; Kalil & Florescu, 2009; Kutza *et al.*, 1998; Lao *et al.*, 1997; Limaye *et al.*, 2008; Mutimer *et al.*, 1997; Portela *et al.*, 1995; Razonable *et al.*, 2001; Reinke *et al.*, 1994a, 1994b) and the inflammatory cytokine TNF has been proposed to drive reactivation through NF-κB-mediated activation of the major immediate early promoter (MIEP) (Döcke *et al.*, 1994; Fietze *et al.*, 1994; Prösch *et al.*, 1995; Stein *et al.*, 1993). However, due to the species specificity of HCMV, it has not been possible to test this hypothesis in the context of organ transplantation. Murine cytomegalovirus (MCMV) is very similar to HCMV in many important ways, including: (i) ability to establish latency and to reactivate; (ii) hierarchical regulation of viral gene expression; (iii) structure, function and organization of the major immediate early (IE) genes (Keil *et al.*, 1987a, b; Meier & Stinski, 2013; Stenberg *et al.*, 1984); (iv) repression of IE gene expression during latency by heterochromatinization of viral genomes (Grzimek *et al.*, 2001; Hummel & Abecassis, 2002; Hummel *et al.*, 2001; Kurz *et al.*, 1999; Liu *et al.*, 2008; 2010; Reeves & Sinclair, 2013; Reeves *et al.*, 2005; Seckert *et al.*, 2013); (v) the presence of similar regulatory elements (e.g. NF-κB, AP-1, Sp1 and CREB/ATF binding sites) in the viral IE enhancers (Liu *et al.*, 2013; Meier & Stinski, 2013); and (vi) (re)activation of the MIEP and/or IE transcription in response to inflammatory mediators or allogeneic stimulation (Döcke *et al.*, 1994; Fietze *et al.*, 1994; Huang *et al.*, 2012; Hummel & Abecassis, 2002; Hummel *et al.*, 2001; Kew *et al.*, 2014; Lee *et al.*, 2004; Liu *et al.*, 2013; O'Connor & Murphy, 2012; Prösch *et al.*, 1995; Reeves & Compton, 2011; Simon *et al.*, 2005; Stein *et al.*, 1993).

Because the IE genes are the first set of genes expressed in lytic infection (Mocarski *et al.*, 2007), and are required to activate the viral replication programme, transcriptional reactivation of IE gene expression is likely a key event in reactivation of the virus. We previously developed a renal transplant model for reactivation of MCMV IE gene expression (Hummel *et al.*, 2001). In this model, transplantation of latently infected kidneys into allogeneic recipients induced IE gene expression and epigenetic reprogramming of the MIEP within 48 h (Hummel *et al.*, 2001; Liu *et al.*, 2013). In addition, allogeneic transplantation induced expression of a *lacZ* reporter transgene under the control of the HCMV MIEP (MIEP-*lacZ*) (Hummel *et al.*, 2001). Although TNF was sufficient to induce both MCMV IE gene expression and the HCMV MIEP-*lacZ* reporter, it was not required to activate IE gene expression in response to allogeneic transplantation in either of these models (Zhang *et al.*, 2008, 2009). We therefore hypothesized that multiple factors may contribute to reactivation of IE gene expression in the complex environment of an allogeneic transplant. The goal of the present study is to identify additional candidates that may contribute to reactivation of IE gene expression in allogeneic transplants, with the long-term goal of developing novel therapeutic approaches to preventing reactivation of the virus. In addition to the TNF/NF-κB and IL-6/mitogen-activated protein kinase (MAPK) signalling pathways, which have been previously implicated in reactivation of HCMV (Döcke *et al.*, 1994; Fietze *et al.*, 1994; Hargett & Shenk, 2010; Huang *et al.*, 2012; Kew *et al.*, 2014; O'Connor & Murphy, 2012; Prösch *et al.*, 1995; Reeves & Compton, 2011; Stein *et al.*, 1993), we have identified novel signalling pathways that have not to our knowledge been previously associated with reactivation of HCMV. Our results suggest that it may be necessary to target multiple signalling pathways to prevent transcriptional reactivation of CMV.

RESULTS

Multiple NF-κB and AP-1 family members are rapidly activated by renal transplantation

The MCMV MIEP is strikingly enriched in NF-κB and AP-1 binding sites (Dorsch-Häsler *et al.*, 1985; Seckert *et al.*, 2013). NF-κB consists of homo- and heterodimeric complexes of p65/RelA, p50, p52, c-rel and RelB subunits (reviewed by Oeckinghaus *et al.*, 2011). Canonical NF-κB (p65/p50) activates transcription of immune response genes in response to inflammatory mediators, including TNF, IL-1β, Toll-like receptor ligands and oxidative stress. Non-canonical NF-κB (p52/RelB) is activated by CD40L, BAFF and lymphotoxin beta. AP-1 is a dimeric complex of Fos, Jun, Maf and ATF family members. Different complexes of this diverse family have varying roles in cellular proliferation, inflammatory immune response and oxidative stress (Eferl & Wagner, 2003; Hernandez *et al.*, 2008; Karin, 1995; Karin & Shaulian, 2001; Shaulian & Karin, 2002).

We previously demonstrated that NF-κB p50/p65 and the junD component of AP-1 were activated within 48 h after renal transplantation and that transcription factor activation correlated with reactivation of IE expression (Hummel *et al.*, 2001; Zhang *et al.*, 2008). Here, we have expanded our analysis to include earlier times and additional NF-κB and AP-1 family members (Fig. 1). Latently infected kidneys were transplanted into allogeneic recipients and analysed at 3, 24 or 48 h. Multiple members of the AP-1 family were rapidly activated by transplantation but they differed in the kinetics of subsequent inactivation. c-Fos was strongly, but transiently, activated; junD was also strongly activated at 3 h, and this activation was sustained through 48 h, albeit at lower levels; c-jun was rapidly activated, but was no longer active at 48 h. No activation of JunB, FosB, Fra-1 or Fra-2 was observed at any time point (data not shown). Both the p65/RelA and RelB subunits of NF-κB were activated within 3 h and remained activated at 24 and 48 h, but RelB activity increased between 3 and 24 h, while p65 activity remained constant.

NF-κB and AP-1 family members are differentially regulated in allogeneic transplants

Transcription factor activity is regulated at multiple levels, including RNA transcription and stability, as well

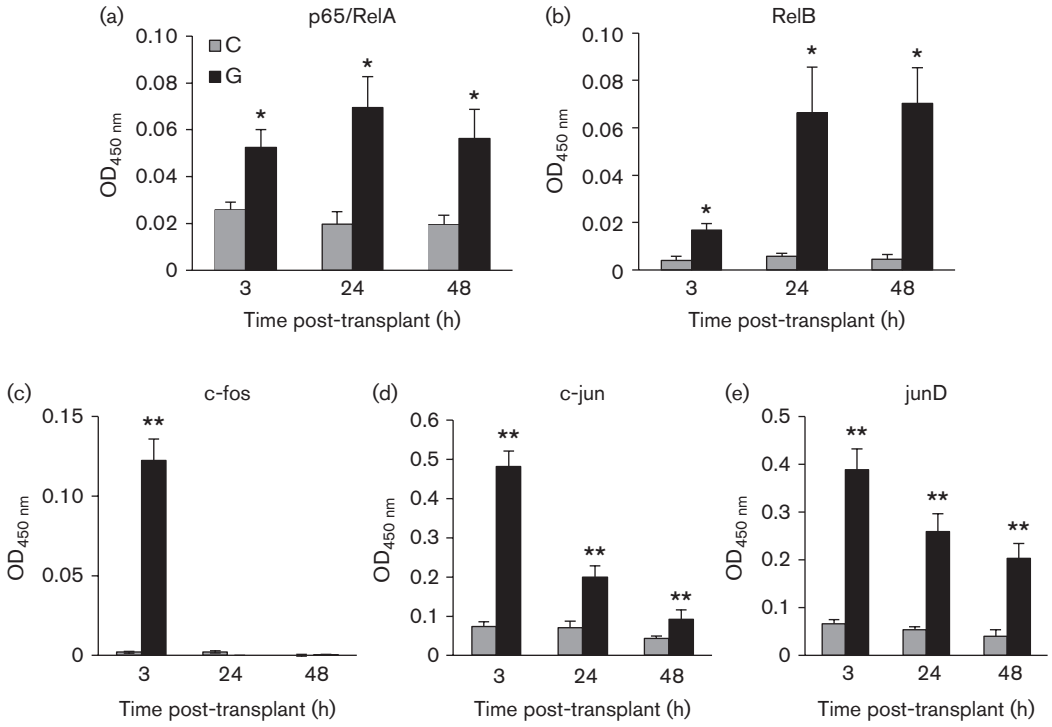


Fig. 1. Transcription factor activation induced by allogeneic transplantation. Kidneys from latently infected BALB/c mice were transplanted into allogeneic C57BL/6 recipients and harvested 3, 24 or 48 h post-transplant (G), as indicated on the *x*-axis. The contralateral latent donor kidney (C) was harvested at the time of the transplant as a matching Day 0 control. Nuclear extracts were prepared and analysed by TransAm assays, $n=5$/time point. Results are expressed as the mean plus standard error. $^{**}P<0.01$; $^{*}P<0.05$.

as protein localization, stability and post-translational modification. To gain insight into the mechanisms controlling changes in activity of NF-κB and AP-1 family members, we analysed expression of RNAs encoding these proteins (Fig. 2). The results show that expression of p65/RelA was upregulated at 3 h post-transplant, then fell below control levels at 24 h and returned to basal levels by 48 h. Expression of RelB peaked at 3 h, but in contrast to p65/RelA, its expression remained elevated relative to controls at 24 and 48 h. These results suggest that the increased activity of NF-κB family members observed in allogeneic transplants (Fig. 1) may be due in part to increased RNA expression. NF-κB activity is controlled by a complex regulatory circuit (Ruland, 2011). Iκ *Balpha* is an inhibitory subunit that sequesters the active p65/p50 complex in the cytoplasm and its expression is induced by NF-κB as part of a negative feedback loop to prevent damage associated with prolonged expression of inflammatory genes controlled by NF-κB. Consistent with these observations, Iκ B alpha expression was rapidly, but transiently, induced by transplantation (Fig. 2).

Expression of AP-1 family members was also differentially regulated in allogeneic transplants (Fig. 2). Expression of c-jun and c-fos RNAs were unchanged at 3 h, but were sharply downregulated 24 and 48 h after transplant. The transient increase in activity of c-jun and c-fos at 3 h (Fig. 1) was therefore due to post-transcriptional regulation. In contrast, expression of junD was significantly induced at 3 h post-transplant, and subsequently downregulated.

NF-κB and AP-1 are recruited to the MIEP concomitant with reactivation of IE gene expression

The MCMV MIEP controls expression of two differentially spliced genes, IE-1 and IE-3. IE-3 is the MCMV functional homologue of HCMV IE-2, which is the major transactivator of early gene expression (Angulo *et al.*, 2000; Keil *et al.*, 1987a, 1987b; Martínez *et al.*, 2010; Messerle *et al.*, 1992). We previously showed that allogeneic transplantation induced reactivation of IE gene expression 48 h after transplant, and that RNA polymerase phosphorylated at Ser2 (Pol II pS2) was recruited to both the MIEP and the IE-3 coding region (Hummel *et al.*, 2001; Liu *et al.*, 2013). Here, we show that IE-3 RNA expression was detectable by reverse transcriptase-qPCR at 48 h, but not at 3 or 24 h post-transplant (Fig. 3a and data not shown). To determine whether reactivation of IE gene expression was associated with recruitment of NF-κB and AP-1 to the MIEP, latently infected kidneys were transplanted

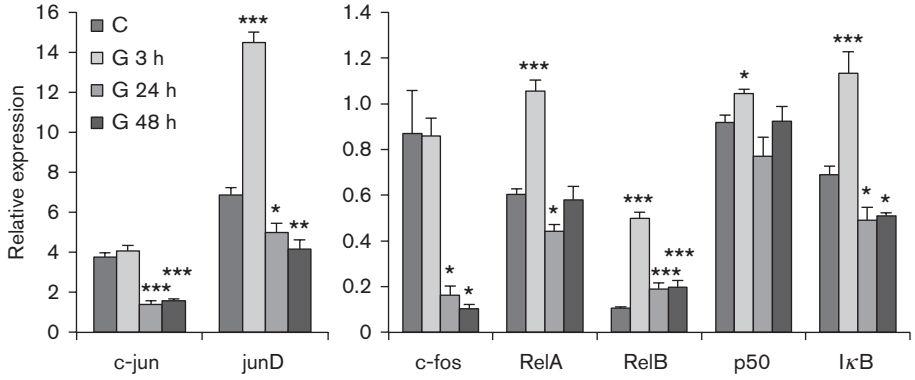


Fig. 2. Expression of transcription factor RNAs in control and kidney allografts. RNA expression was analysed in control (C) kidneys and in grafts harvested 3 (G 3 h), 24 (G 24 h) or 48 h (G 48 h) after transplant and normalized to hypoxanthine phosphoribosyltransferase (HPRT) RNA. Results are expressed as the mean plus standard error. *P* values were determined by comparison of expression in the graft to pooled controls. ***$P<0.001$; **$P<0.01$; *$P<0.05$; $n=4$.

into allogenic recipients and analysed at 48 h by chromatin immunoprecipitation (ChIP) analyses. We analysed binding of histones, Pol II pS2, NF-κB/RelA and p50 and the junD component of AP-1 to the MIEP. Analysis of Pol II and histone modification was published separately (Liu *et al.*, 2013). These results showed that allogeneic transplantation induced major changes in viral chromatin, including recruitment of pS2-Pol II to the MIEP and the downstream IE-3 exon 5, loss of repressive histone marks and gain of activating marks.

Our previous studies showed activation of NF-κB in response to transplantation by analysing binding to oligonucleotides containing consensus NF-κB binding sites (Fig. 1 and Hummel *et al.*, 2001; Zhang *et al.*, 2008). Activation of the transcription factor does not necessarily result in binding to the gene of interest. Here, we show that NF-κB p50 and p65/RelA are in fact bound to the viral MIEP 48 h after transplantation into allogeneic recipients (Fig. 3b). As controls, we show that NF-κB is also bound to the NF-κB-responsive CCL2 promoter (Teferedegne *et al.*, 2006; Wolter *et al.*, 2008), but not to the NF-κB-deficient Hes-1 promoter. In addition, junD was recruited to the MIEP and to the junD-responsive IL-6 promoter (Baccam *et al.*, 2003; Mann *et al.*, 2002; Ndlovu *et al.*, 2009; Smart *et al.*, 2001; Viedt *et al.*, 2002; Zerbini *et al.*, 2003), but not the transcriptionally silent Ant4 promoter (Fig. 3c).

Chromatin remodelling complexes are recruited to the MIEP upon reactivation of IE gene expression

MCMV genomes are highly enriched in histones in latently infected mice, suggesting that the nucleosomes are in a compacted configuration, which is closed to transcription (Liu *et al.*, 2008). Many inducible genes require remodelling of the chromatin by the SWI/SNF complex in order to activate gene expression (Ramirez-Carrozzi *et al.*, 2009). SWI/SNF complexes contain common subunits, including actin, and one of two ATPases, Brahma (BRM) or Brahma-related gene 1 (BRG-1), which confer gene specificity (Kadam & Emerson, 2003; Mohrmann & Verrijzer, 2005). AP-1 family members, including c-jun and c-fos, interact with BRG-1. We therefore analysed recruitment of actin and BRG-1 to the MIEP; there was insufficient material to analyse BRM. Increased binding of actin (Fig. 3d), but not BRG-1 (data not shown), to the MIEP was observed in 48 h allografts. These data indicate that chromatin remodellers may be recruited to the MIEP, although the specific complexes remain to be identified.

Collectively, Figs 1–3 show that allogeneic transplantation induces transcriptional reactivation of IE gene expression and that this correlates with changes in expression, activation and binding of NF-κB and AP-1 transcription factors to the MIEP. In order to develop new strategies that may prevent reactivation of latent CMV, it is important to understand how these transcription factors are activated in the context of transplantation.

Inflammatory mediators are elevated in recipient plasma

Allogeneic transplantation activates both innate and adaptive inflammatory immune responses at early times post-transplant (Einecke *et al.*, 2005; El-Sawy *et al.*, 2004; Famulski *et al.*, 2007, 2006; Hummel *et al.*, 2009). Transcriptional reactivation of IE gene expression and remodelling of viral chromatin may be mediated by activation of inflammatory signalling pathways induced by interaction of ligands in the plasma, which are elevated in response to allogeneic transplantation, with receptors expressed on cells of the kidney. To identify potential mediators of reactivation, we analysed plasma taken from recipient mice at the time of sacrifice for biomarkers of inflammation. These results showed that several inflammatory cytokines,

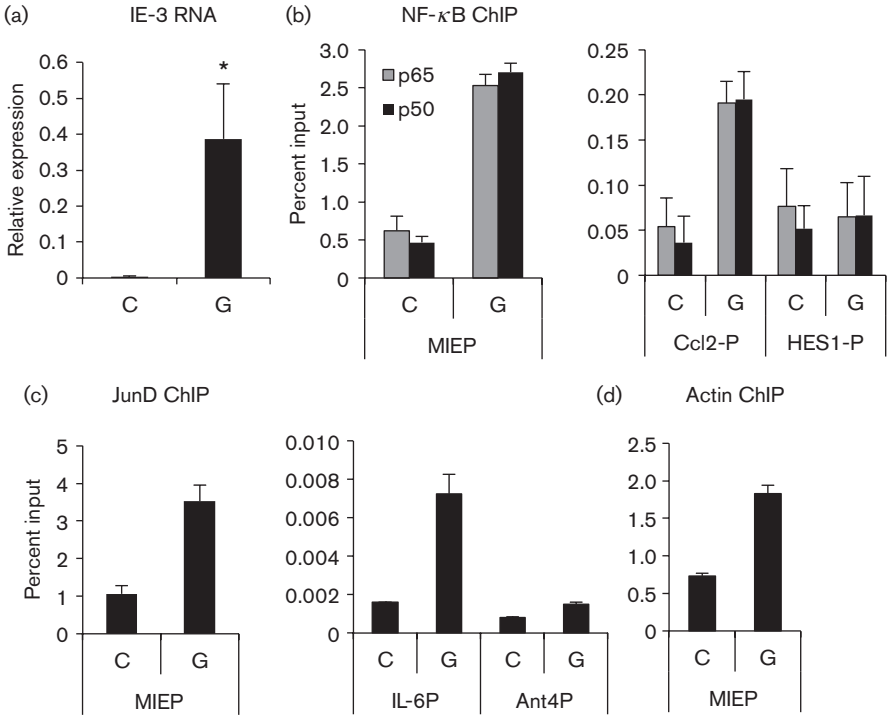


Fig. 3. Reactivation of IE gene expression correlates with recruitment of transcription factors and remodelling of the MIEP. (a) Expression of IE-3 in matching control (C) and transplanted (G) kidneys 48 h after transplant. MCMV IE-3 RNA expression was normalized to expression of cellular Eef2; $n=5$. Error bars show the mean plus standard error. $*P<0.05$. (b) ChIP analysis of p65/RelA and p50 NF-κB subunits bound to the MCMV MIEP (left panel) and to the promoters of the cellular Ccl2 and Hes1 genes (right panel) in pooled chromatin from control (C) or 48 h allografts (G). (c) Binding of junD to the MIEP (left panel) and to the promoters of the IL-6 and Ant4 genes (right panel). (d) Binding of actin to the MIEP. The data shown are representative of at least two independent assays, except NF-κB p50 ($n=1$). Error bars show SD of technical replicates analysed in triplicate.

including IL-1β, IL-18 and IL-6, were elevated relative to controls (Fig. 4). Plasma levels of IL-6 protein were strongly elevated at 3 h, and fell at 24 h (Fig. 4a, b), but were elevated relative to controls at 48 h (Fig. S1, available in the online Supplementary Material). Plasma levels of IL-1 and IL-18 rose more gradually (Fig. 4), but were also elevated relative to controls at 48 h (Fig. S1).

Inflammatory signalling pathways are activated in renal allografts

To further investigate the response to allogeneic transplantation, we analysed global changes in gene expression within the transplanted kidney by microarray and Ingenuity Pathway Analysis. As controls, we analysed RNA from the contralateral donor kidneys removed at the time of the transplant. The results (Fig. 5a) showed that TNFR1, TNFR2, IL-1, CD40 and IL-6 signalling, as well as their downstream effector pathways, including NF-κB, JAK/STAT and ERK/MAPK, were differentially regulated at multiple time points. The heat map showing expression of the individual members of these pathways (Fig. 5b) illustrates the complex and rapidly evolving nature of the inflammatory response induced by allogeneic transplantation. Five patterns of expression were apparent in the genes in these signalling pathways: genes in Cluster I were induced 24–48 h after transplant; genes in Cluster II were induced at 3 h and subsequently downregulated; genes in Cluster III were rapidly downregulated and returned to near baseline levels after 24 h; genes in Cluster IV were rapidly downregulated and recovered expression more slowly, while genes in Cluster V were slowly downregulated and remained below baseline at 48 h. Mapk9/JNK2, which phosphorylates and activates c-jun (Jaeschke *et al.*, 2006; Gupta *et al.*, 1996), was among the genes rapidly downregulated at 3 h. Genes induced at 3 h included genes involved in both positive (Tnfrsf1b, IL1R1, TLR4, p65/RelA, Nfkb2/p100, RelB, Map3k14/NIK, IRAK, Ube2v1, Peli1, Bcl10) and negative (Nfkbia/IκB alpha, Tnfaip3/A20) regulation of the NF-κB signalling pathway (Oeckinghaus *et al.*, 2011; Ruland, 2011). Increased expression of these negative regulators may account for the decreased expression of some genes in this pathway observed at 24–48 h. Positive (NF-IL6/CEBPβ, STAT3) and negative (SOCS3, Map2k6) regulators of IL-6 signalling (Akira *et al.*, 1990; Bode *et al.*, 2001;

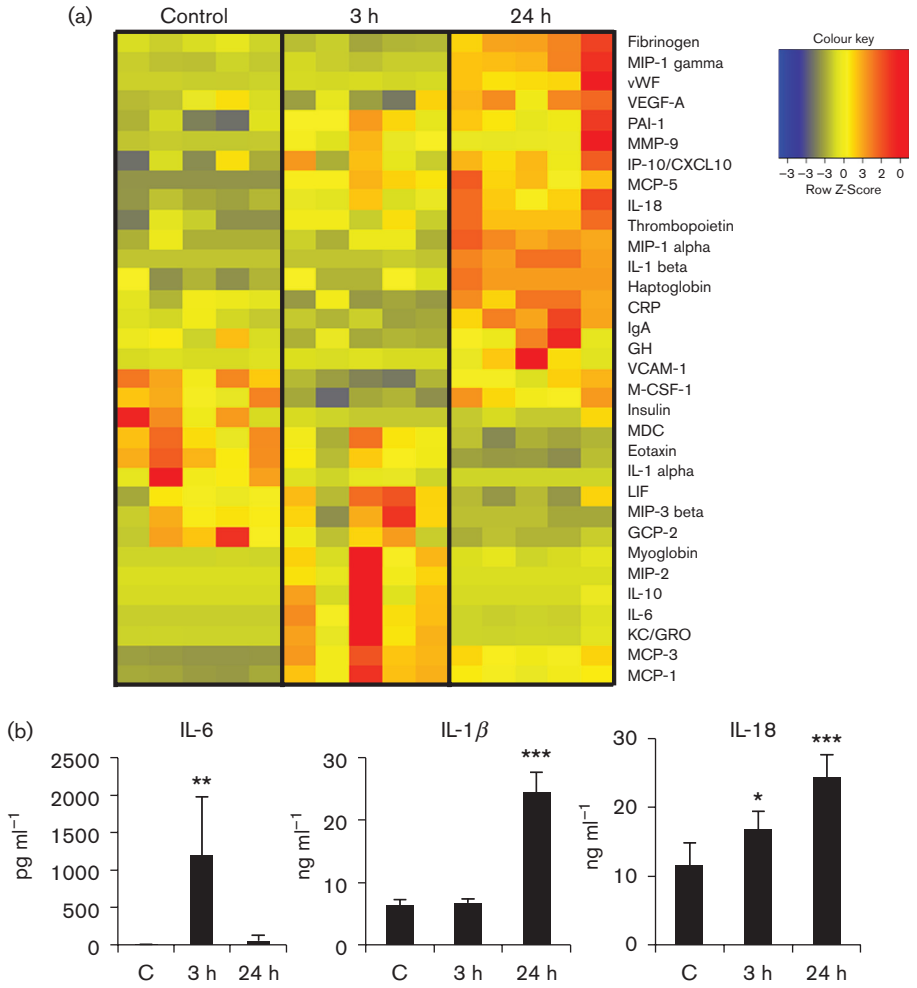


Fig. 4. Allogeneic transplantation induces release of inflammatory proteins into the plasma. (a) Heat map of differentially regulated plasma proteins in control (C) and recipient mice at 3 and 24 h after transplant, $n=5$/group. (b) Graphical representation of the plasma protein values of selected cytokines. Results are expressed as the mean plus SD. ***$P<0.001$, **$P<0.01$, *$P<0.05$.

Heinrich *et al.*, 2003) were also strongly upregulated at 3 h. IL-6 expression itself did not meet the threshold for statistical significance in the microarray analysis, due to sample variability, but IL-6 expression was significantly induced in the same RNA samples when analysed by the more quantitative method of RT-qPCR (Fig. 6). Upregulation of TNF expression in response to allotransplantation was also confirmed by RT-qPCR (Fig. 6). In contrast to IL-6, which was upregulated rapidly and transiently, TNF expression increased gradually from 3 to 48 h.

IL-6 and IL-1β proteins were also elevated in plasma of recipient mice (Fig. 4), indicating a good correspondence between the presence of the ligand in the plasma and the response of the cells in the kidney. CD40 was not included among the plasma analytes, and thus, a correlation between the ligand and receptor signalling could not be determined for this factor. Despite increased expression of TNF RNA in the allografts (Fig. 6) and pathway analysis showing upregulation of TNFR1 and TNFR2 signalling (Fig. 5), TNF protein was not detected in the plasma above threshold levels. This may be due to rapid turnover of TNF protein (plasma $t_{1/2}=6$–7 min) (Beutler *et al.*, 1985), or to dilution of the protein in recipient plasma.

DISCUSSION

CMV genomes are heterochromatinized and the IE genes, which are required for lytic replication, are transcriptionally silent in latent infection (Grzimek *et al.*, 2001; Hummel *et al.*, 2001; Kurz *et al.*, 1997, 1999; Liu *et al.*, 2008, 2010; Seckert *et al.*, 2013; Simon *et al.*, 2005). Activation of IE gene expression, through recruitment of transcription factors that control MIEP activity and remodelling of viral chromatin, is therefore likely required for reactivation of

the virus. The MIEP has multiple potential binding sites for AP-1 and NF-κB (Hummel *et al.*, 2001; Seckert *et al.*, 2013). Here, we show that several members of these transcription factor families are rapidly activated and bound to the MIEP following allogeneic transplantation.

Interestingly, individual family members showed dynamic changes in the kinetics of activation/inactivation, as well as changes in RNA expression. c-fos was rapidly and transiently activated following transplantation (Fig. 1). c-fos is an unstable protein whose expression and activity is regulated at multiple levels (Sasaki *et al.*, 2006). Activation of c-jun was also rapidly induced, but in contrast to c-fos, retained some activity relative to controls at 24 h. Loss of c-jun activity may be due in part to the rapid downregulation of Mapk9/JNK2 (Fig. 5b), which activates and phosphorylates c-jun (Jaeschke *et al.*, 2006). In addition, expression of c-jun/c-fos RNA was strongly downregulated by 48 h (Fig. 2). This result is consistent with their roles as early response genes whose RNAs are rapidly expressed in response to stimuli and quickly degraded due to recognition of an AU-rich RNA decay element in the 3′ UTR (Chen & Shyu, 1995). While c-jun and c-fos are well-known oncoproteins with roles in cellular proliferation, junD is atypical of other AP-1 family members in both regulation of gene expression and in biological function (Hernandez *et al.*, 2008). JunD is activated by oxidative stress, including renal ischaemia/reperfusion injury (Kim *et al.*, 2005) and protects cells against apoptosis through upregulation of genes that mitigate oxidative damage (Gerald *et al.*, 2004; Hernandez *et al.*, 2008; Lamb *et al.*, 2003; Pillebout *et al.*, 2003; Tsuji, 2005). As with c-jun and c-fos, junD was rapidly activated by transplantation, but, in contrast to these proteins, junD activity was sustained for 48 h (Fig. 1 and Hummel *et al.*, 2001; Zhang *et al.*, 2008) and downregulation of the RNA relative to controls was less dramatic.

Both p65/RelA and RelB NF-κB family members were rapidly activated by allogeneic transplantation, and remained active at 48 h (Fig. 1), despite downregulation of the RNAs and increased expression of negative regulatory molecules, including Nfkbia (Iκ Balpha) at 3 h and Nfkbie at 48 h (Figs 2 and 5b). While many studies have focused on canonical signalling pathways leading to activation of p65/RelA, non-canonical pathways leading to activation of RelB have not to our knowledge been previously implicated in reactivation of CMV. The relative roles of different NF-κB family members in regulation of the MIEP requires further investigation. Collectively, the data in Figs 1, 2 and 5 show that MIEP-regulatory transcription factor expression and activity following transplant is regulated by complex and dynamic processes, which are controlled both transcriptionally and post-transcriptionally.

We previously demonstrated that allogeneic transplantation induces transcriptional activation of IE gene expression, and epigenetic reprogramming of MCMV chromatin, including a switch from heterochromatic to euchromatic histones and recruitment of RNA polymerase II to the MIEP (Hummel *et al.*, 2001; Liu *et al.*, 2013). Here we show that transcription factors thought to be important in regulation of IE gene expression, including the canonical NF-κB subunits p50 and p65/RelA and the junD component of AP-1, are bound to the MIEP in response to allogeneic transplantation. To our knowledge, this is the first demonstration that these transcription factors are not only activated by stimuli that induce reactivation; they are in fact recruited to the viral DNA concomitant with reactivation of IE gene expression. Though not definitive, these correlative results strongly suggest that these factors play an important role in activating expression of the IE genes.

In this respect, the MCMV MIEP is similar to other viral enhancers, which mimic inflammatory immune response

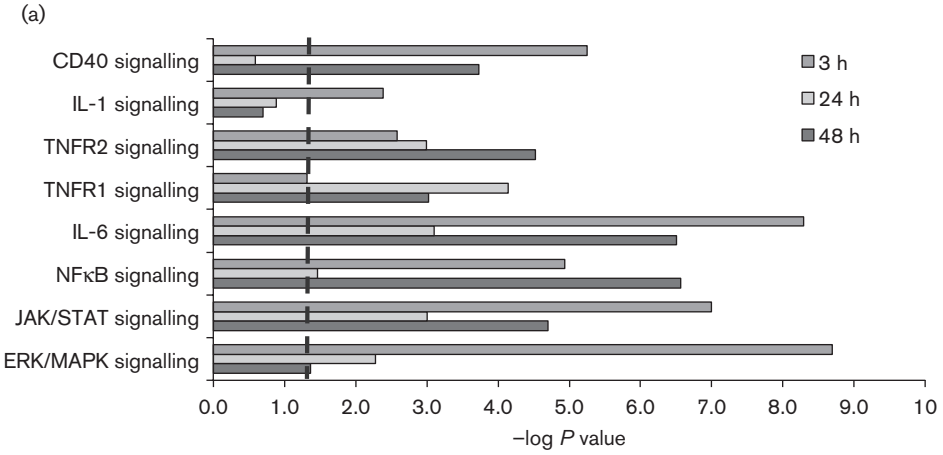
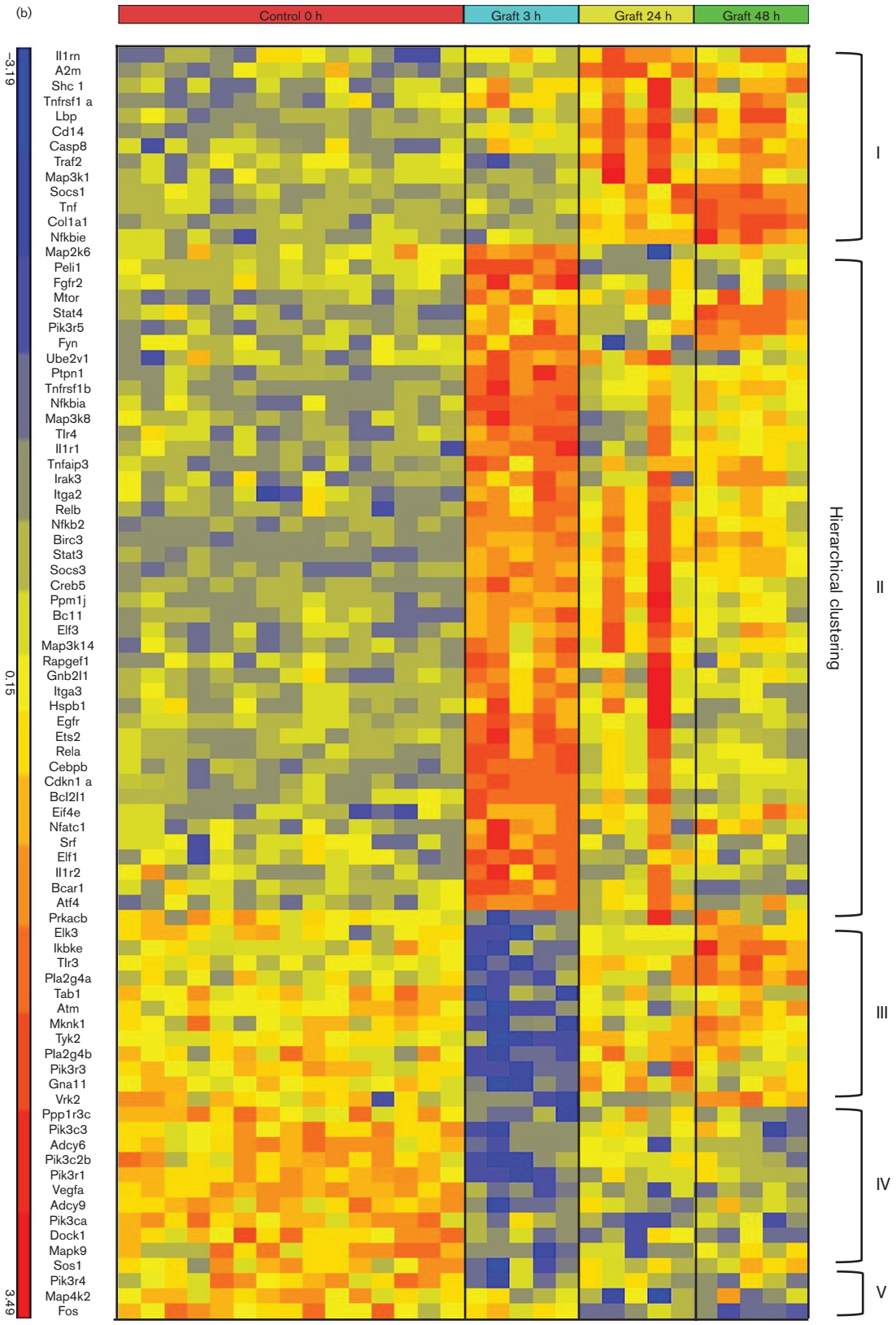


Fig. 5. Dynamic changes in inflammatory signalling pathways are induced by allogeneic transplantation. (a) Ingenuity Pathway Analysis of selected signalling pathways upregulated by allogeneic transplantation at 3, 24 and 48 h. Pathways with −log *P* value >1.3 (dashed line) are statistically significant. (b) Heat map of RNA expression of genes within these pathways determined by microarray analysis.

Fig. 5. (cont.)


(b)
Control 0 h
Graft 3 h
Graft 24 h
Graft 48 h
−3.19
0.15
3.49
Il1rn
A2m
Shc 1
Tnfrsf1 a
Lbp
Cd14
Casp8
Traf2
Map3k1
Socs1
Tnf
Col1a1
Nfkbie
Map2k6
Peli1
Fgfr2
Mtor
Stat4
Pik3r5
Fyn
Ube2v1
Ptpn1
Tnfrsf1b
Nfkbia
Map3k8
Tlr4
Il1r1
Tnfaip3
Irak3
Itga2
Relb
Nfkb2
Birc3
Stat3
Socs3
Creb5
Ppm1j
Bc11
Elf3
Map3k14
Rapgef1
Gnb2l1
Itga3
Hspb1
Egfr
Ets2
Rela
Cebpb
Cdkn1 a
Bcl2l1
Eif4e
Nfatc1
Srf
Elf1
Il1r2
Bcar1
Atf4
Prkacb
Elk3
Ikbke
Tlr3
Pla2g4a
Tab1
Atm
Mknk1
Tyk2
Pla2g4b
Pik3r3
Gna11
Vrk2
Ppp1r3c
Pik3c3
Adcy6
Pik3c2b
Pik3r1
Vegfa
Adcy9
Pik3ca
Dock1
Mapk9
Sos1
Pik3r4
Map4k2
Fos
Hierarchical clustering
I
II
III
IV
V

genes (Kropp *et al.*, 2014). Cellular inflammatory response genes have been divided into two classes, primary and secondary, based on their requirement for new protein synthesis (Hargreaves *et al.*, 2009; Medzhitov & Horng, 2009; Ramirez-Carrozzi *et al.*, 2009, 2006). Primary response genes are rapidly inducible, due to constitutive binding of RNA polymerase to nucleosome-free promoters, and elongation of transcription is the rate-determining step in activating expression of these genes. Conversely, the promoter regions of secondary response genes are covered by nucleosomes, and remodelling of the chromatin is required for binding of RNA polymerase to activate transcription. Our studies show that binding of RNA polymerase to the MIEP is not detectable in latent mice and that, in addition to RNA polymerase and transcription factors, actin, a component of many chromatin-remodelling complexes, is recruited to the MIEP following transplantation. Thus, regulation of the MIEP may be more similar to secondary immune response genes.

Remodelling complexes can alter the configuration of the chromatin through: (i) nucleosome sliding, in which the position of the nucleosome on the DNA changes; (ii) remodelling, in which histones remain bound but the DNA becomes more accessible; (iii) ejection of nucleosomes from the DNA; and (iv) replacement of canonical histones with a variant histone (Mohrmann & Verrijzer, 2005). We previously observed no change in binding of histone H3 to the MIEP in allografts (Liu *et al.*, 2013). These results therefore suggest that remodelling does not occur through eviction.

Current antiviral therapies target viral DNA replication in cells in which reactivation of latent genomes has already occurred. An alternative strategy, which would be less susceptible to viral escape mutants, would be to target cellular pathways that lead to transcriptional reactivation of IE gene expression. A greater understanding of the molecular mechanisms leading to activation of the MIEP is required to realize this goal. TNF expression is upregulated by allogeneic transplantation (Fig. 6 and Hummel *et al.*, 2009, 2001) and TNF is sufficient to activate MCMV IE gene expression and/or reactivation *in vivo* (Cook *et al.*, 2006; Hummel *et al.*, 2001; Simon *et al.*, 2005). However, our previous studies showed that TNF was not required for reactivation of IE gene expression in response to allogeneic transplantation (Zhang *et al.*, 2009). These results suggest that multiple factors may contribute to transcriptional reactivation of viral gene expression in the context of allogeneic transplantation. To identify additional factors, we performed plasma proteomic analysis and transcriptional profiling of genes that were differentially expressed following allogeneic transplantation. While many pathways are activated in the complex environment of an allogeneic transplant, our studies identified five extracellular ligands that may contribute to reactivation of IE gene expression and reprogramming of viral chromatin. These include TNF, IL-1 and IL-18, which activate canonical NF-κB through engagement of their cell surface receptors, and

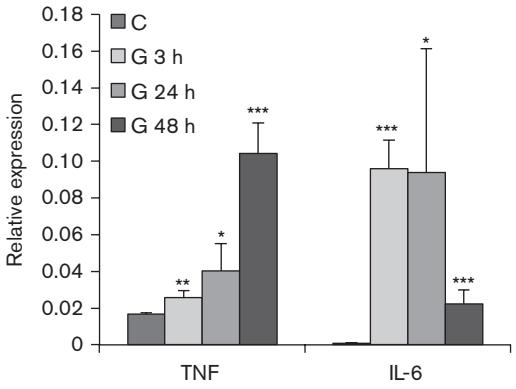


Fig. 6. Expression of TNF and IL-6 is induced in kidney allografts. RNA expression was analysed in control (C) kidneys and in grafts harvested 3 (G 3 h), 24 (G 24 h) or 48 h (G 48 h) after transplant and normalized to HPRT RNA. Results are expressed as the mean plus standard error. *P* values were determined by comparison of expression in the graft to pooled controls. ***$P<0.001$; **$P<0.01$; *$P<0.05$; $n=4$.

CD40/CD40L, which activates both canonical and non-canonical NF-κB (Pomerantz & Baltimore, 2002). These pathways also activate AP-1, through activation of JNK-mediated phosphorylation of jun family members (Quezada *et al.*, 2004; Weber *et al.*, 2010; Wullaert *et al.*, 2006). In addition, we found elevated levels of IL-6 in the plasma, increased IL-6 expression and activation of IL-6 signalling pathways in the kidney. Recent studies indicate that IL-6 activates the HCMV MIEP in monocyte-derived dendritic cells through recruitment of CREB (cAMP response element-binding protein) and MSK (mitogen- and stress-activated protein kinase)-mediated histone phosphorylation (Kew *et al.*, 2014; Reeves & Compton, 2011). Although reactivation of the MIEP may occur through different pathways in different models, our results suggest that IL-6 may also have a role in reprogramming of viral chromatin in transplant-induced reactivation of CMV.

The alloimmune response to a transplanted organ is initiated by non-specific injury resulting from mitochondrial damage, oxidative stress and release of 'danger signals' from damaged tissue (de Groot & Rauen, 2007; Gallucci *et al.*, 1999; Kono & Rock, 2008). These signals induce maturation of antigen-presenting cells in the graft that migrate to the lymph nodes and activate the adaptive arm of the immune response. NF-κB and AP-1 are activated by ischaemia/reperfusion injury and oxidative stress as well as inflammatory cytokines (Gloire *et al.*, 2006; Kamata *et al.*, 2005; Karin, 1995; Karin & Shaulian, 2001; Kim *et al.*, 2005; Morgan & Liu, 2011; Oeckinghaus *et al.*, 2011; Shaulian & Karin, 2002). In our studies, we observed very rapid activation of NF-κB and AP-1 family members, and biphasic activation of some pathways, including CD40, IL-6, NF-κB and JAK/STAT signalling

(Fig. 5a). These observations suggest that different factors may contribute to activation of NF-κB and AP-1 at different times in the rapidly evolving environment of an allogeneic transplant, and that it may be necessary to target mediators of intracellular damage, such as reactive oxygen species, as well as extracellular ligands, to prevent reactivation of IE gene expression. Identification of the signalling pathways that lead to reactivation and the factors that activate these pathways will be the focus of future studies.

Allograft rejection, sepsis, acute illness, IL-6 and TNF have long been implicated in reactivation of HCMV in patients (Cook *et al.*, 1998; Döcke *et al.*, 1994; Fietze *et al.*, 1994; Grattan *et al.*, 1989; Heininger *et al.*, 2001; Hibberd *et al.*, 1992; Kalil & Florescu, 2009; Kutza *et al.*, 1998; Lao *et al.*, 1997; Limaye *et al.*, 2008; Mutimer *et al.*, 1997; Portela *et al.*, 1995; Razonable *et al.*, 2001; Reinke *et al.*, 1994a) and allogeneic stimulation, TNF, IL-6 and lipopolysaccharide have been shown to induce reactivation of HCMV in experimental models (Hargett & Shenk, 2010; Huang *et al.*, 2012; Kew *et al.*, 2014; O'Connor & Murphy, 2012; Reeves & Compton, 2011; Söderberg-Nauclér *et al.*, 1997). Allogeneic transplantation and TNF are sufficient to activate both an HCMV MIEP-*lacZ* transgene and MCMV IE gene expression (Hummel *et al.*, 2001). Thus, there is considerable convergence between clinical studies of patients infected with HCMV, experimental models of HCMV latency/reactivation and animal studies with MCMV. Identification of the factors that induce reactivation of MCMV IE gene expression may therefore eventually lead to new approaches to prevent reactivation of HCMV and its sequelae.

METHODS

Mice and transplants. BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MCMV (Smith strain) was purchased from the American Type Culture Collection, and propagated in mice by harvesting salivary glands 14 days post-infection. Virus stocks were titrated on confluent monolayers of murine embryo fibroblasts. To establish latency, 3–4-week old female BALB/c mice were infected by intraperitoneal injection with 5×10^5 p.f.u. of MCMV (Smith strain) and housed for 3–6 months in the Northwestern University Center for Comparative Medicine. Donor kidneys from latently infected BALB/c mice were transplanted into recipient C57BL/6 mice as previously described (Zhang *et al.*, 1995), except that recipients were bilaterally nephrectomized at the time of the transplant. The pararenal glands were left intact. The contralateral donor kidneys were frozen in liquid nitrogen at the time of the transplant for use as matching, Day 0 latent controls. Transplanted kidneys were frozen in liquid nitrogen at the time of sacrifice, and recipient plasma was collected for analysis of plasma proteins. Recipients were sacrificed at 3, 24 or 48 h, as indicated in the text. These studies were approved by the Northwestern University Institutional Animal Care and Use Committee and conducted accordingly.

Transcription factor activation. Analysis of transcription factors was performed on nuclear extracts isolated from frozen kidney tissue using TransAm kits as directed by the manufacturer (Active Motif). Nuclear extract (10 or 5 μg) was used to analyse NF-κB or AP-1 family members, respectively.

ChIP analyses. Transplanted kidneys were harvested at 48 h for ChIP analysis. The contralateral donor kidneys were removed at the time of the transplant for use as latent controls. Fresh tissue was finely minced on ice, and the chromatin was fixed in 1 % formaldehyde as previously described (Liu *et al.*, 2008, 2010), and frozen in liquid nitrogen. Due to the very low MCMV DNA copy number in kidneys of latent mice [$\sim$1 copy of MCMV DNA per 10 000 cellular genomes (Li *et al.*, 2012)] and the large amount of chromatin required for each ChIP, chromatin from 5–6 kidneys was pooled for each antibody. Frozen pellets of fixed tissue from 30–50 transplants were resuspended in hypotonic lysis buffer, and processed for immunoprecipitation as previously described (Liu *et al.*, 2008, 2010). Ten per cent of the chromatin was removed for analysis of input DNA. The remaining chromatin was pre-cleared with IgG and incubated overnight at 4 °C with antibodies against target proteins as previously described (Liu *et al.*, 2008, 2010). The following antibodies were used: NF-κBp65 (Abcam, Ab7970-1), NF-κBp50 (Abcam Ab7971), junD (SantaCruz Biotech, sc-74x) and actin (SantaCruz Biotech, sc-7210). qPCR analysis to quantify input and immunoprecipitated DNA were performed as previously described (Liu *et al.*, 2008, 2010).

Plasma protein analysis. Plasma (70 μl) from transplant recipients or untreated C57BL/6 control mice was frozen in liquid nitrogen and analysed for inflammatory proteins at MyriadRBM by Luminex using the Rodent MAPv3.1 platform.

Transcriptome analysis. Frozen tissue was immediately transferred to tubes containing TriZol and 5 mm stainless steel beads (Qiagen) and the tissue was disrupted by mechanical shaking in a TissueLyser (Qiagen) at room temperature for 5 min. RNAs were purified with PureLink RNA Minikits (Ambion), using on-column DNase treatment as directed by the manufacturer. RNA was quantified on a nanodrop spectrophotometer and quality was assessed on an Agilent 2100 bioanalyser. Genome-wide RNA expression was analysed using Affymetrix MoGene-2_1-st-v1 microarrays.

RT-qPCR. cDNAs were synthesized from 2 μg total cell RNA using a High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems) for analysis of cellular gene expression. Gene-specific RNAs were amplified from 10 ng cDNA under standard conditions with the following TaqMan Gene Expression Assays (Applied Biosystems): TNF, Mm00443260_g1; RelA (p65), Mm00501346_m1; Nfkb1 (p50), Mm00476361_m1; RelB, Mm00485664_m1; c-jun, Mm00495062_s1; c-fos, Mm00487425_m1; junD, Mm04208316_s1; Nfkbib/IkBalpha, Mm00456849_m1. Relative gene expression was determined using hypoxanthine phosphoribosyltransferase (HPRT) RNA (Mm01545399_m1) as the internal control. MCMV IE-3 expression was analysed as previously described (Liu *et al.*, 2008).

Statistical analysis. To identify changes in plasma protein expression, the MyriadRBM data were first filtered by excluding analytes in which >75 % of the measurements were below the detection threshold. Fisher's exact test was performed to ensure that none of the excluded proteins was significantly more likely to be undetectable in one group than another. The filtered multi-analyte protein MAP data were first log-transformed for better approximation to normal distribution and then analysed by a linear model, in which the common variance is estimated with the pooled data per protein wise. *P* values were obtained by performing *t*-test between the conditions for each protein, and then adjusted by Benjamini-Hochberg false discovery rate procedure for multiple comparisons. Changes in expression were visualized with a heat map generated in R.

For genome-wide RNA expression, microarrays were normalized using Robust Multichip Average (RMA) (Bolstad *et al.*, 2003) and

signal filters of Log2 <5.69 were used to exclude probe sets with low signal intensities. Pairwise class comparisons were carried out using a one-way ANOVA by the Method of Moments (Eisenhart, 1947) in Partek Genomics Suite 6.6. A False Discovery Rate (FDR) of $<5\,\%$ was used for all class comparisons. Pathway mapping to biologically significant pathways was done using Ingenuity Pathway Analysis. All pathways were adjusted using the Benjamini-Hochberg correction for multiple testing. The microarray expression data from the study were deposited at the NIH Gene Expression Omnibus (GEO) website under the accession number GSE 72392.

Student's *t*-test was used to determine statistical significance for expression of individual RNAs and plasma proteins. $P<0.05$ was considered statistically significant.

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