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Molecular Mechanisms Mediating Activation of the Cytomegalovirus Major Immediate Early Enhancer in Allogeneic transplantation

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ABSTRACT

Molecular mechanisms mediating activation of the cytomegalovirus major immediate early enhancer in allogeneic transplantation

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Reactivation of latent human cytomegalovirus is of significant concern in immunocompromised transplant patients. However, the mechanisms controlling reactivation of latent CMV have not been understood. It is likely that reactivation is initiated by induction of IE gene expression. In this thesis, I studied molecular mechanisms leading to transcriptional activation of IE gene expression in vivo. Using transgenic mice carrying a beta-galactosidase reporter gene under the control of the HCMV major immediate early enhancer and deficient in the TNF receptors, I demonstrated that signaling through TNF receptors plays a critical role in activation of the HCMV enhancer in allogeneic kidney transplantation. This occurred primarily through TNFR2. Furthermore, I demonstrated that signaling through the TNF receptors is required for activation of NF-κB in allogeneic transplantation. The JunD component of AP-1 was also activated very strongly in allogeneic transplants, but this activation occurred independently of signaling through the TNF receptors.

In addition, I studied the role of oxidative stress induced by renal ischemia/reperfusion injury (I/R) in activation of the enhancer. Allogeneic transplantation induces nonspecific injury due to deprivation of oxygen resulting from interruption of blood flow (ischemia) and subsequent restoration of blood flow (reperfusion injury) as well as injury due to immune recognition of foreign antigens. I demonstrated that renal

ischemia/reperfusion injury (I/R) activates the HCMV enhancer independently of TNF. Induction of MIEP-lacZ expression was preceded by weak and transient activation of NF-κB and strong and sustained activation of AP-1, especially JunD. Activation of transcription factors was preceded by TNFR-independent formation of reactive oxygen species (ROS). These results are consistent with the hypothesis that TNF-independent signaling due to oxidative stress induced by I/R injury can activate the HCMV enhancer through ROS-mediated activation of AP-1.

My results suggest that activation of either the TNF signaling pathway or the MAP kinase signaling pathway can activate the enhancer, and targeting these pathways may have therapeutic value in patients with CMV infection.

ACKNOWLEDGEMENTS

Dedicated to my loving parents and sister.

I want to thank my mentors-Mike and Mary-and everyone in my lab- Zhigao, Laura, Gail, Shixian, Thomas, and Jenny for all their guidance and help, and to all my friends.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. CMV

CMV is a ubiquitous herpesvirus which is present in the majority of adults. Most people contract CMV infection during early childhood or adolescence. Human cytomegalovirus (HCMV), like all other members of the herpesvirus group, establishes life-long persistence after primary infection. In immunocompetent hosts, both primary CMV infection and subsequent reactivation is relatively benign, and often subclinical [1]. In contrast, primary infection and reactivation can be associated with serious morbidity or mortality in immunocompromised patients, including organ transplant recipients, bone marrow transplant recipients, patients receiving immunosuppressive drugs, and HIV-infected patients. Severe disease can develop in immunologically immature children. CMV is the most important cause of congenital viral infection in the United States. The common manifestions of CMV disease are pneumonitis, retinitis, and gastrointestinal disease [2]. Infection of a seronegative women during pregnancy can result in intrauterine transmission of CMV to the fetus, resulting in death of the fetus or CMV related complications after birth, including hearing loss, visual impairment, or diminished mental and motor capabilities.

CMV disease occurs at a high frequency among CMV-negative patients who receive allografts from a CMV-positive donor [3]. In addition, CMV infection increases the risk of chronic rejection and the incidence of other opportunistic infections [4]. Thus, CMV infection, even when diagnosed and treated, results in appreciable morbidity and adds

significantly to the overall cost of patient care. However, the cell types harboring latent virus, the definition of latency at a molecular level, and the mechanisms leading to reactivation from latency are not well understood [1].

1.1.1 Virus Structure and genome organization

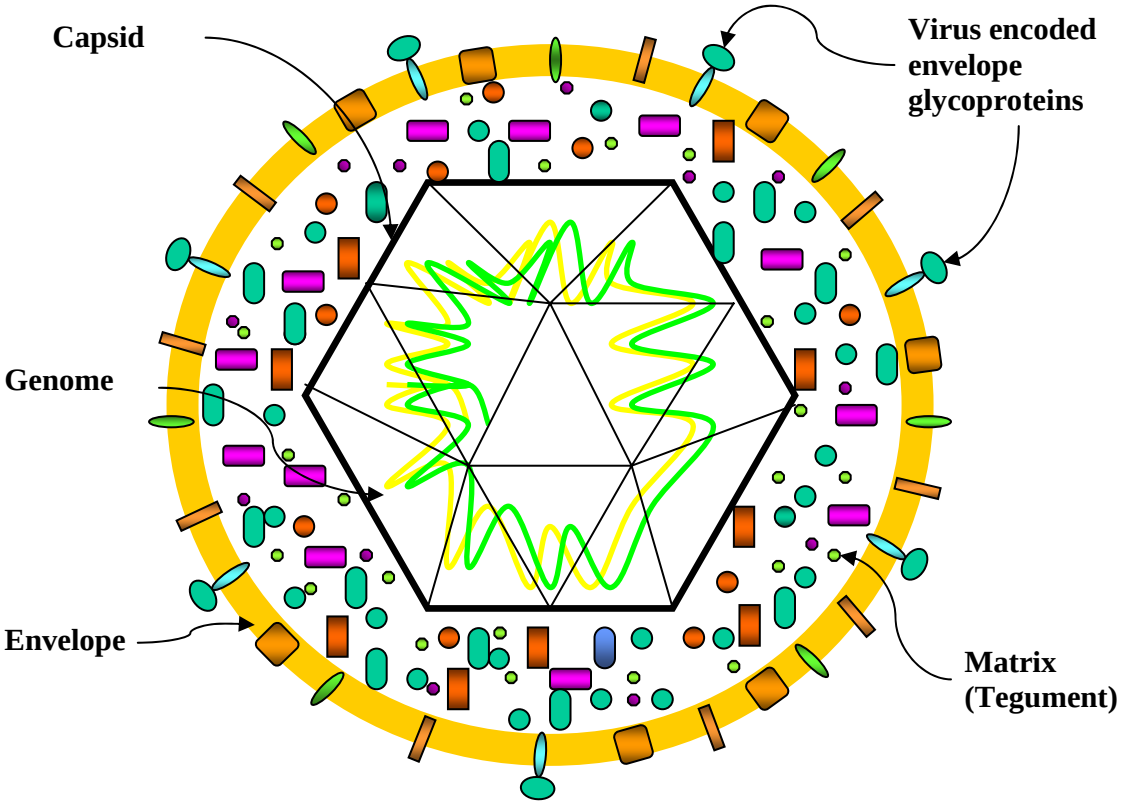
CMVs have a virion structure typical of herpesviruses (Fig. 1). The virion consists of a large double-stranded linear DNA-containing core in a 100nm diameter icosahedral capsid that is surrounded by a tegument or matrix. These components are enclosed in a lipid bilayer envelope carrying a large number of virus-encoded glycoproteins (Fig. 1). Mature virions range in size from 150 to 200nm. CMVs have the largest genomes of any of the herpesviruses, ranging from 180 kilobase pairs (kbp) for equine CMV to 230 to 240 kbp for human, simian, guinea pig, and murine CMVs [1].

1.1.2. Characteristics of CMV

1.1.2.1 Betaherpesviruses

Although CMV shares many characteristics with other herpesviruses, including virion and genome structure and the ability to establish persistent and latent infections, CMV has distinguishing characteristics, such as salivary gland tropism, species specificity, and slow growth in cultured cells. These differentiating characteristics were initially used to define the betaherpesviruses, of which CMV is the prototypical member. The other members of the betaherpesviruse family are human herpes virus type 6 (HHV6) and type 7 (HHV7) which were discovered relatively recently. Both of these viruses have been associated with various neurological complications such as encephalitis [5]. CMV related viruses infect a wide range of animal hosts, including humans, other

Figure 1: Virus structure of CMV. The virion of human CMV consists of an icosahedral capsid encasing a 235-kbp linear genome, surrounded by a tegument or matrix and enveloped in a lipid bilayer carrying a large number of virus-encoded glycoproteins.


Capsid
Virus encoded
envelope
glycoproteins
Genome
Envelope
Matrix
(Tegument)

primates, domestic animals, and rodents, but each virus is species specific. Therefore, animal models have been used to study pathogenesis of HCMV in vivo. Murine CMV (MCMV), which is similar to HCMV with respect to genome organization, pathogenesis, regulation of gene expression and ability to establish latent infection and to reactivate, has been used by many investigators as a model to study CMV pathogenesis. Transgenic mice carring a β-galactosidase reporter gene under the control of the HCMV IE promoter/enhancer have also been used as a model to study regulation of HCMV IE gene expression in vivo [6].

1.1.2.2 Cell tropism and excretion during acute infection

During active HCMV infection and recovery from illness, the virus replicates productively in many cell types including epithelial cells, endothelial cells, fibroblasts, leukocytes circulating in the peripheral blood and specialized parenchymal cells, including smooth muscle cells in the gastrointestinal tract and hepatocytes. Virus can be excreted in the urine, saliva and breast milk, confirming the involvement of a number of tissues as sites of productive infection and viral transmission during active infection [1]. Following the acute phase of infection, CMV establishes a lifelong latent infection like other herpesviruses.

1.1.3. Virus growth cycle

Relative to other herpesviruses, the replication cycle of CMV is slow, requiring 48 to 72 hr for human CMV (24 to 36 hr for murine CMV) to begin the release of progeny virus. Heparan sulfate proteoglycans (HSPGs) that are expressed on the surface

of most cell types, mediate tethering of the virus to the cell membrane [7]. Attachment and penetration are mediated by integrins and the EGF receptor [8-10]. It has been shown that acute infection of CMV activates several cellular signaling pathways, leading to activation of transcription factors that are important in regulating IE gene expression. CMV is known to rapidly induce activation of nuclear factor kappaB (NF-κB) after infection of primary fibroblasts and monocyte-derived cell lines [11-16]. Several different pathways have been proposed for activation of NF-κB, including signaling through CD 14 and TLR2 and activation of PI3K [17, 18]. Additionally, activation of G-protein-dependent signaling pathways by CMV phosphorylates and activates cytosolic phopholipase A_2 ($cPLA_2$), and subsequently activates the arachidonic acid cascade, which generates intracellular ROS and leads to activation of NF-κB in smooth muscle cells [19]. HCMV infection of quiescent fibroblasts was shown to induce activation of mitogen-activated protein kinase (MAPK) kinase 1 and 2 (MKK1/2), resulting in activation of two of their downstream targets, extracellular signal regulated kinase 1 and 2 (ERK1/2) [20]. In addition to NF-κB, up-regulation of Sp1 was observed shortly after the binding of HCMV to the cell surface [21]. Activation of both NF-κB and Sp1 occur independently of viral replication since they can be activated by UV-inactivated virus or by the viral envelope glycoprotein gB alone.

During productive infection, CMV gene expression follows a coordinated program typical of herpesviruses. This program has been operationally split into three phases, termed immediate early (IE), early and late (Fig. 2). The IE genes are expressed immediately upon viral entry; expression of these genes does not depend on the expression of any other viral genes. Cellular signaling pathways activated by the virus

and by viral tegument proteins such as pp71, the viral transactivator protein, help to activate IE gene expression during productive infection [1]. IE products, in turn, lead to expression of the early genes, while shutting off their own expression. Early viral gene products are generally involved in viral DNA replication, and expression of these genes permits replication of viral DNA and subsequent late gene expression. Late genes encode the viral structural proteins. Expression of these genes allows viral assembly and production of new viral particles. Thus, induction of IE gene expression is essential for all aspects of virus production and may be negatively regulated during latent infection [1].

1.1.4 Regulation of IE gene expression

1.1.4.1 Immediate early genes (IE gene)

Four regions of IE gene expression have been mapped on the human CMV genome. The overwhelming majority of these IE transcripts arise from the MIE locus located between 169 and 175 kbp on the human CMV genome [1]. In all CMVs, two prominent IE gene transcripts arise by differential splicing of a single transcription unit encoded by the MIE locus. In human CMVs, the most abundantly expressed IE genes are IE1, which is the product of exons 1,2,3, and 4, and IE2, which is produced from a spliced transcript composed of exons 1,2,3, and 5 (Fig. 3). The first three exons (1,2,3) encoding the 15 amino-terminal amino acids are thus shared by IE1 and IE2 transcripts. While IE1 can cooperate with IE2 to activate IE1/IE2 promoter-enhancer expression and to drive expression of viral Early and Late genes, overexpression of IE1 alone is sufficient to influence gene expression via a variety of cellular transcription factors. IE1 activates NF-κB [13, 22]. IE1 binds to retinoblastoma (Rb) family member p107

Figure 2: CMV gene expression. Productive replication follows the coordinate expression of ordered sets of viral genes. Gene expression may be divided into sequentially expressed kinetic classes: α (immediate early), β (early), γ (late) based on time of synthesis after infection. Thus, the overall complexity of the regulatory cascade has similarities to other herpesviruses in that viral functions expressed during the earliest phases of the replication cycle play regulatory roles in determining the timing of the cascade.

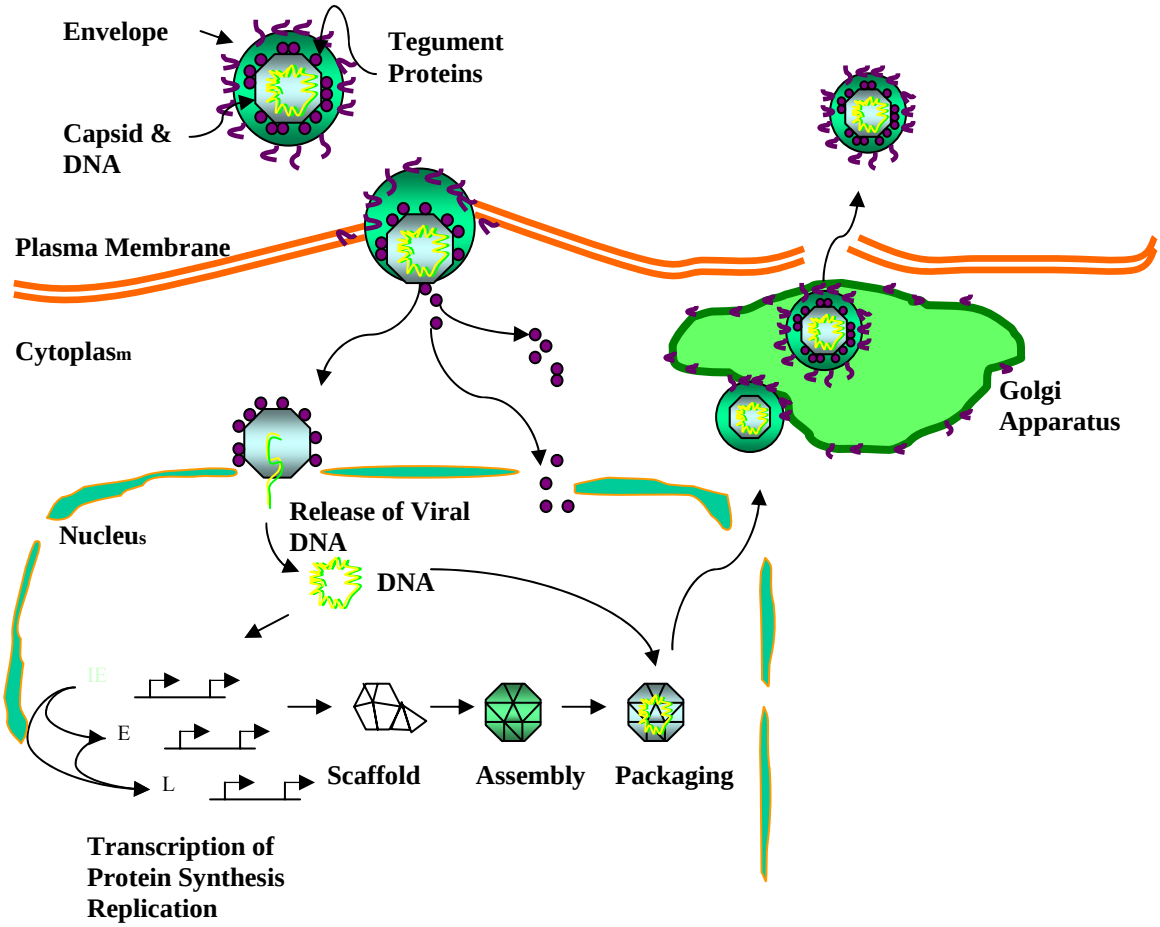
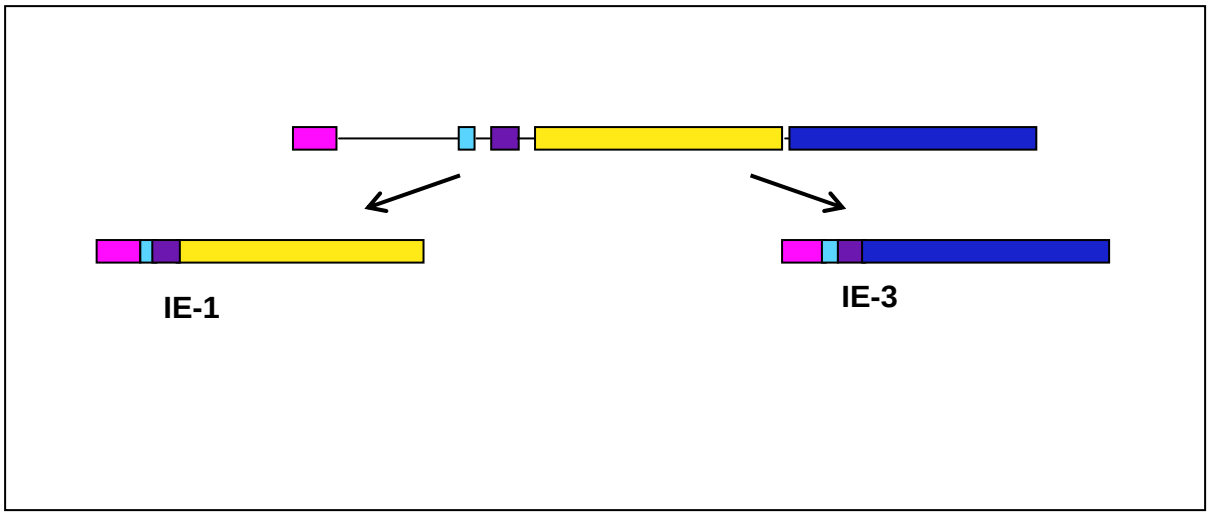

Envelope
Tegument Proteins
Capsid & DNA
Plasma Membrane
Cytoplasm
Golgi Apparatus
Nucleus
Release of Viral DNA
DNA
IE
E
L
Scaffold
Assembly
Packaging
Transcription of
Protein Synthesis
Replication

Figure 3: Schematic of the DNA encoding the MCMV IE region and IE-1 and IE-3 transcripts[4,5]. Exons are indicated as colored boxes.


IE-1
IE-3

and its binding is sufficient to overcome p107-mediated cell growth suppression [23, 24]. IE1 can upregulate the activity of AP-1. AP-1 dependent gene expression is induced via activation of the MAP kinase/extracellular signal-regulated kinase pathway [25]. IE2 is the primary viral regulatory protein controlling the switch from IE to Early and Late gene expression during productive infection [1]. IE2 also down-regulates IE1/IE2 gene expression by binding to a cis-repression signal (crs) located near the start site of transcription of either gene and altering RNA polymerase II preinitiation complex formation [1]. IE2 alone or with IE1 regulates different promoters via a number of different transcription factors. It targets the basal transcription apparatus through interaction with TBP and TFIID and through potential stabilization of TFIID-associated factors such as TAFII130 and TAFII110 [26-31]. The two genes that are similar to human CMV IE1 and IE2 are referred to as IE1 and IE3, respectively, in murine CMV [1].

1.1.4.2 MIE locus

IE gene expression is controlled by the major immediate early promoter/enhancer (MIEP/E) which is located between -550 and +1 relative to the transcription start site. This region is a highly complex regulatory region and contains multiple elements that interact with many host transcription factors. The HCMV MIEP/E consists of repeats of 18-, 19-, 16-, and 21bp motifs (Fig. 4) [32]. There are four 18-bp repeat elements containing consensus NF-κB/rel binding sites, five 19-bp repeat elements containing consensus or near-consensus CREB/activating transcription factor (ATF) binding sites, and three 21-bp repeats containing YY1, Ets.2 repressor factor, and Sp-1 sites. In one study, the 16-bp repeats were shown to bind NF-1 [33]. In addition, there are retinoic acid receptor elements

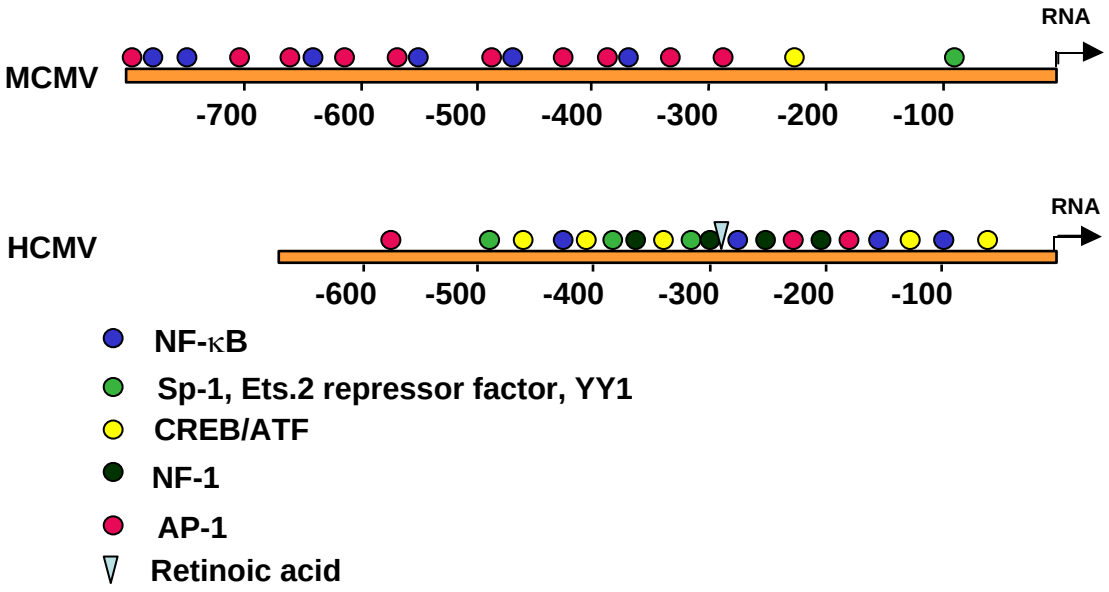
(RARE) which bind to retinoic acid receptor complexes, three AP-1 binding sites and one serum response factor [32]. The MCMV promoter/enhancer, which controls the expression of MCMV IE gene, has five sites matching the AP-1 consensus site, four additional AP-1 sites juxtaposed to classic NF-κB sites, and an AP-1 site paired with an inverted NF-κB site (Fig. 4) [34]. Although the specific arrangements of the transcription factor binding sites differ among the human, simian, murine and rat CMVs, the enhancers of these CMVs also contain multiple copies of binding sites for NF-κB, AP-1, or ATF.

The functional role of the MIEP enhancer was investigated using enhancerless MCMV bacterial artificial chromosome (BAC) genomes. This enhancerless MCMV is severely deficient in virus synthesis when transfected into murine fibroblasts, demonstrating that enhancer is important for MCMV growth [35]. When the human CMV enhancer was substituted for its murine counterpart in MCMV, the human CMV enhancer supported full replication in cell culture [35] as well as in lethally irradiated mice [36]. These observations suggest that these diverse enhancers function in a similar way.

Several studies have addressed the function of specific transcription factor binding sites. Early studies showed that the 18-bp repeats can bind to NF-κB and transient transfection assays using reporter constructs showed that the synthetic 18-bp repeats are sufficient to make the gene responsive to IE-1 transactivation [11]. More recent studies have shown that p65 subunit of NF-κB is sufficient to activate the enhancer and that c-jun can cooperate with NF-κB to drive the activation of IE enhancer to higher levels. In addition, LPS was shown to activate the enhancer in an NF-κB dependent manner [37]. These studies suggest that both NF-κB and AP-1 can contribute to the

Figure 4: Putative transcription factor binding sites in the MCMV and HCMV *ie1* promoter/enhancer regions. Binding sites in the MCMV promoter/enhancer are based on analysis of the nucleotide sequence. Binding sites in the HCMV promoter/enhancer are based on previous analyses [32].

Transcription factor binding sites in the MIEP/E



activation of the HCMV enhancer.

The role of the 19-bp repeats containing CREB/ATF binding sites has also been investigated using transient transfection assays. The results showed that the 19-bp repeats are sufficient to mediate the stimulation of the enhancer in response to mitogen. In addition, pp71, the viral transactivator protein, was able to drive the activation of HCMV enhancer through the ATF sites [38-40]. These studies suggested that CREB/ATF binding sites, in addition to the NF-κB sites, are important for regulation of the HCMV enhancer.

More recently, development of BAC technology has allowed mutational analysis of specific transcription factor binding sites in the context of the viral genome [41]. Surprisingly, mutation of the five19-bp-repeats to abolish CREB/ATF binding had no effect on HCMV replication in fibroblasts. However, these sites were important in stimulation of HCMV replication in response to the elevation of cAMP [42]. These results suggest that although activation of CREB is sufficient to drive reporter gene expression, other signaling pathway activated by the virus during infection might be able to substitute for CREB in activation of IE gene expression.

Although previous studies suggested that NF-κB is important in regulation of MIEP/E gene expression, there has been no mutational analysis of virus to study the functional importance of 18-bp-repeats within the context of the virus. The Yurochko group showed that proteasome inhibitors or aspirin, which block activation of NF-κB, block activation of IE gene expression during infection, suggesting a requirement for NF-κB in viral replication [43]. However, mutation of the NF-κB sites in these enhancer-swap

viruses in which the MCMV enhancer was replaced by the HCMV enhancer demonstrated that deletion of NF-κB sites has no effect on viral replication [44]. As these two studies conflict each other, it is not clear whether NF-κB is required or not for viral replication.

Isomura et.al investigated the role of the Sp1 sites in the virus [45]. Both Sp1 and Sp3 are ubiquitously expressed members of the Sp1 family. It has been shown that Sp1 can activate a large number of genes involved in cellular processes such as cell cycle regulation, chromatin remodeling, and the propagation of methylation-free CpG islands [46, 47]. Sp3 has been found to act as a transcriptional activator at Sp1-like sites on many promoters. Either Sp1 or Sp3 transcription factors are able to bind to GC boxes in HCMV proximal enhancer [45]. Deletion of one of the two Sp1/Sp3 binding sites in the MIEP/E did not significantly change viral gene expression or viral replication in fibroblast cells. In contrast, mutation of both sites caused significant impairment of viral gene expression and replication. This suggests that either Sp1 or Sp3 play an important role in HCMV replication [45].

Finally, the Zhu group identified two IFN-γ response elements in the MIE promoter/enhancer region. In addition, they showed that IFN-γ induced the expression of IE1 and IE2, and that virus mutants with deletions or point mutation of these elements were significantly compromised in viral replication. This result suggests that IFN-γ is important for regulation of IE gene expression [48].

The 21-bp element has been found to bind to the transcription factor YY1. YY1 represses MIE promoter activity in non-permissive, undifferentiated teratocarcinoma cell

(NTera2 cells) and monocyte THP-1 cells, but not in differentiated cells [49, 50]. Co-transfection of IE reporter plasmids in the presence of YY1-expressing plasmids in undifferentiated and differentiated NTera2 cells showed that YY1 strongly inhibits CAT activity in undifferentiated NTera2 cells. Deletion of these specific binding sites from major IE promoter/reporter constructs results in increased IE promoter activity in normally non-permissive cells [49]. YY1 expression is decreased in differentiated cells [49]. These studies suggest that YY1 may be a negative regulator of IE gene expression which is lost during cell differentiation. However, deletion of the 21-bp repeats and the modulator from the HCMV genome does not significantly affect transcription from the MIEP/E in infected human fibroblasts or undifferentiated NTera2 cells and monocytic THP-1 cells [51]. Based on these studies, YY1 might be important to maintain latency.

1.2 CMV latency and reactivation

1.2.1 CMV latency

After primary infection in the host, the virus establishes a life-long state of latency from which it reactivates periodically. Although no molecular definition of CMV latency exists, latency has been defined operationally as the inability to detect infectious virus despite the presence of viral DNA. The state of viral gene expression in latently infected tissues has been controversial. It has been unclear whether a true state of latency exists in which there is no expression of viral genes associated with productive infection or whether there is a low level of persistent infection. If there is a true latency, then reactivation results from a change in the transcriptional program of the latently infected cell which would result in viral replication. Alternatively, in the case of persistent infection, reactivation results from failure of the immune system to control infected cells.

These key questions regarding the nature of latency and reactivation have been difficult to answer due to the difficulty in obtaining clinical samples for analysis, the limited number of cell types harboring viral DNA, the low viral copy numbers in latently infected individuals, and the lack of good *in vitro* model systems for studying latency and reactivation.

Three lines of evidence suggest that there may be persistent infection. First, IE-1 transcripts have been observed in some organs from some latently infected mice [52-56]. Since IE-1 expression is associated with productive infection, detection of IE-1 transcripts suggests the presence of infected cells. Second, immunosuppression experiments with animal models of CMV have shown that reactivation can be induced by treatment of animals with immunosuppressive therapies, such as total body irradiation or administration of cytotoxic drugs such as cyclophosphamide or azathioprine, or by imunodepletion of T cells or T cell subsets, suggesting that productively infected cells are present in latently infected animals [52, 57-60]. Lastly, high levels of HCMV-specific CD8+ T cells are detectable in seropositive individuals long after resolution of primary infection [61-65]. In MCMV infection, virus-specific memory T cells accumulate steadily over time [66]. These results suggest that memory T cell populations are formed by continuous or repetitive exposure to antigen and persistent infection is likely to occur in CMV latency.

However, other studies have suggested that there is a true latency. Expression of IE genes is not detectable in naturally HCMV infected human CD34+ cells or in monocytes from healthy seropositive donors. IE gene expression can be induced in these cells under some circumstances (see below), indicating that lack of detection is not due to lack of sensitivity [67].

Furthermore, a recent study showed that the latent DNA load in the lungs of mice latently infected with MCMV was approximately 2,000 copies per 10^6 lung cells, which is equivalent to 600,000 copies. However, about 1 out of 60,000 latent viral genomes was transcriptionally active at the MIE locus [68]. The very low frequency of cells expressing IE transcripts as opposed to these carrying viral DNA suggests that expression of IE-1 transcripts is not an inherent feature of latency and that detection of IE gene expression is due to reactivation of the virus in a small population of the cells carrying viral DNA. Taken together, these studies support the view that, in an individual, most cells that carry viral DNA are truely latent. In these cells, expression of viral genes associated with productive infection is silent. However, within that same individual there is frequently a small number of cells in which reactivation has occurred. Since immunosuppressive therapies are sufficient to induce reactivation, immune surveillance is important in controlling infection once reactivation has begun.

1.2.2. Sites of latency

Although studies of the sites for carriage of HCMV during latency are limited, in vivo studies of both HCMV and MCMV latency suggest multiple sites of latency for both organ and cell types. HCMV DNA can be detected in CD14+ peripheral blood mononuclear cells and in CD34+ cells, which are the progenitors for B cells, T cells, and monocyte/macrophage lineage [69-72]. However, infectious virus is not detectable in the blood.Granulocyte-macrophage progenitor (GMPs) cells derived from fetal liver cells can be infected with HCMV *in vitro.* Although HCMV DNA is detectable in these cells, viral replication was not detected at 3 or 4 weeks postinfection. Virus can be reactivated from these cells, indicating that the viral DNA is competent for replication. This study

suggests that GMPs may be a reservoir of latent CMV infection in vivo [73]. Sinusoidal endothelial cells also have been suggested as a possible site of latent infection. HCMV can be detected in endothelial cells during acute infection [74]. Endothelial cells can be infected with HCMV in vitro [75]. Some studies have detected HCMV DNA-positive epithelial, endothelial and smooth muscle cells in the absence of viral protein expression, suggesting these cell types could be additional reservoirs of latent virus [76]. However, further studies are required to explore this possibility.

In mice latently infected with MCMV, viral DNA is detected by PCR-in situ hybridization (PISH) in bone marrow cells, in endothelial cells in multiple organs (kidney, spleen, liver, heart) and in macrophages (lung) [56, 77]. Additional studies, which show that MCMV can be reactivated by coculturing macrophages from latently infected mice with murine embryo fibroblasts (MEFs), also support the view that macrophages are a site of latency [78]. Taken together, studies of both HCMV and MCMV latency indicates that cells of the monocyte/macrophage lineage derived from bone marrow cells and endothelial cells may be sites for latent infection. Whether there are additional sites for latent cytomegalovirus, however, is unknown. The mechanisms leading to establishment and maintenance of HCMV latency are also unknown.

1.2.3 Reactivation of CMV

Since there is controversy regarding the state of latency, the mechanism of reactivation is not well understood. The observations of the Sinclair group, which show that IE gene expression is not detectable in monocyte or CD34+ cells from seropositive individuals, but can be induced by differentiation of these cells into mature dendritic cells, supports the view that there is a true latency and that reactivation is induced by

transcriptional activation of IE gene expression. In addition, studies on the frequency of cells expressing IE transcripts as opposed to those carrying viral DNA are consistent with this model. Since IE gene expression is required for productive infection and IE gene expression is infrequently detected among cells carrying viral DNA, it is likely that induction of IE gene expression is the first step of reactivation. Two models have been proposed to explain how IE gene expression is induced during reactivation: 1) that induction of IE gene expression is tied to diffentiation of latently infected hematopoietic progenitor cells into macrophages or dendritic cells; 2) that transcriptional activation of IE gene expression occurs as a result of an inflammatory immune response.

1.2.3.1. Cellular differentiation

Early studies showed that NTera2 cells, derived from a human teratocarcinoma, are not permissive to HCMV [79]. However, treatment of these cells with retinoic acid (RA), a derivative of vitamin A, prior to infection induces differentiation of these cells into a neuronal phenotype and also makes them permissive to HCMV infection [80, 81].The block in infection was found to be due to inability to express the IE genes and treatment with RA was found to release this block. Transfection studies with reporter constructs showed that treatment of NTera2 cells with RA could activate the HCMV enhancer through RA responsive elements (RARE) [80]. In addition, overexpression of RA receptor (RAR) was able to activate an MIEP/E reporter construct [80]. Upon differentiation, expression of the transcription factor YY1 disappeared, suggesting that the down-regulation of HCMV IE1 by YY1 is abolished in differentiated NTera2 cells [49]. However, treatment with RA after infection does not induce reactivation [82].

Several studies have shown that monocytes are also non-permissive for infection due to a block in IE expression. Differentiation of monocytes into macrophages makes these cells fully permissive to HCMV infection in vitro [49, 79-81, 83-87]. Sinclair's lab has used CD34+ hematopoetic stem cells or monocytes from healthy, HCMV seropositive individuals to study reactivation of HCMV. Very recently, they demonstrated that differentiation of naturally infected CD34+ hematopoetic progenitor cells or of monocytes into mature DC induces transcriptional activation of IE gene expression and reactivation of latent HCMV [67]. They also showed that reactivation is associated with changes in acetylation of the histones associated with the MIEP. A significant decrease in the level of histone deacetylase1 was also observed. This study is consistent with previous studies suggesting that histone deacetylases are involved in repression of the MIEP in non-permissive NTera-2 cells [82, 88]. These findings suggest that reactivation of virus from latency may occur as a result of differentiation-induced changes in histones associated with the MIEP.

1.2.3.2. Inflammatory immune response

Previous studies have indicated that the allogeneic response could be important in inducing reactivation from latency. In animal models, transplantation of organs from latently infected donors into allogeneic recipients combined with immunosuppression gives rise to reactivation of CMV. Additional studies showed that reactivation of virus could be induced by tissue implantation, administration of allogeneic cells as well as blood transfusion in combination with immunosuppression [57, 89-92]. Recently, Jay Nelson's group showed that allogeneic stimulation of peripheral blood mononuclear cells

(PBMC) from naturally infected healthy individuals, induces differentiation of monocytes into macophages and reactivates latent virus [93, 94]. Clinically, reactivation of CMV is frequently associated with rejection of the transplanted organ or with other conditions accompanied by high levels of inflammatory cytokines, such as graft-versus-host diseases, cirrhosis, and sepsis [95-99]. Allograft rejection or allogeneic stimulation induces an inflammatory immune response, releasing cytokines which activate transcription factors thought to be important in regulation of the MIEP, including NF-κB and AP-1 [100-106].

Treatment with anti-T cell antibodies to control acute rejection, which induces release of TNF, is a known risk factor for HCMV reactivation[107-110]. This suggests that TNF, an inflammatory cytokine, might be important in reactivation of virus. TNF is known to activate the transcription factor NF-κB (Fig. 5). There are several NF-κB binding sites in the MIEP and TNF has been shown to stimulate the activity of the HCMV IE enhancer/promoter region through activation of NF-κB in transient transfection studies in vitro [111]. Treatment of latent exogenously infected granulocyte-macrophage progenitor cells with TNF also induces reactivation of HCMV [69]. Finally, studies from the Abecassis lab have shown that TNF is sufficient to induce IE gene expression in vivo and that TNF expression is induced in allogeneic transplants prior to activation of IE gene expression [112].

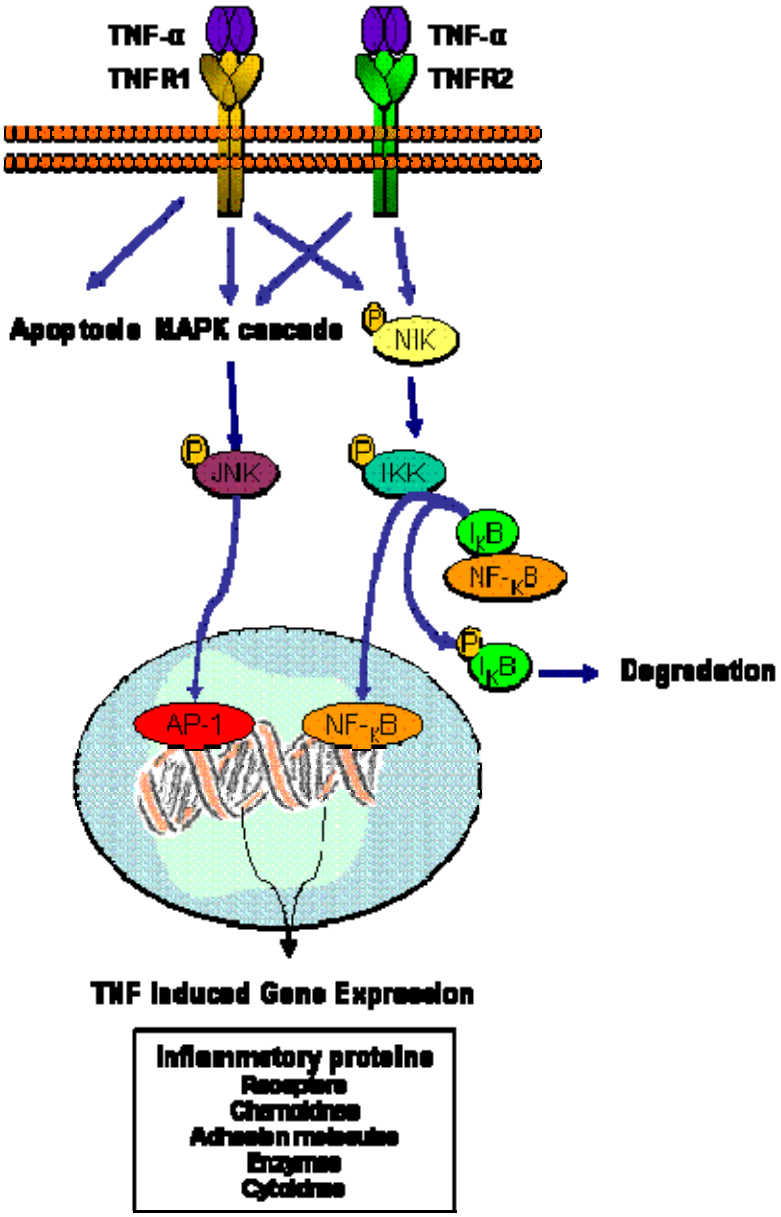
As previously mentioned, differentiation of immature DC into mature DC reactivates virus ex vivo [88]. However, this differentiation is induced by treatment of the cells with a cocktail of cytokines, and inflammatory mediators including LPS and TNF. Taken as a whole, these observations are consistent with the hypothesis that signaling pathways

activated by inflammatory cytokines induced by infection or by an alloimmune response, particularly the TNF signal transduction pathway, could lead to transcriptional activation of IE gene expression. This is likely to occur both as a result of activation of transcription factors that control activation of the MIEP and changes in the configuration of the chromatin which allows access to those transcription factors.

1.3. Previous studies

Previous studies in our lab have shown that no or little MCMV IE gene expression is detectable in control kidneys of MCMV latently infected mice. Allogeneic transplantation of kidneys from latently infected mice into uninfected allogeneic recipients induces MCMV IE-1 gene expression [112]. However, IE3, an alternatively spliced transcript initiated at the IE promoter, E-1, an early gene product, and gB, a late gene product were not detectable in kidneys transplanted into immunocompetent recipients. This suggests that there is a true latency and that reactivation is initiated by transcriptional activation of IE gene expression. Reactivation of infectious virus may require suppression of the host immune response in addition to allostimulation. In addition, activation of the HCMV enhancer was induced by transplanting kidneys from MIEP-lacZ transgenic mice carrying a β-galactosidase reporter gene under the control of the HCMV enhancer into allogeneic recipients. These studies show that both the HCMV and the MCMV enhancers are induced by allogeneic transplantation. This induction was accompanied by increased expression of transcripts encoding inflammatory cytokines, including TNF. In addition, TNF alone is sufficient to induce IE gene expression in some organs [68, 112].

Figure 5: Schematic diagram of TNF signaling. There are two forms of TNF: transmembrane TNF and soluble TNF made by cleavage from a transmembrane precursor. Both form biologically active homotrimers and initiate signaling by binding to TNF receptors. Two families of TNFα receptors (TNFR) have been highly characterized: TNFR1 and TNFR2. TNFR1 can induce apoptosis via its death domain. Alternatively, ligand bound TNFR1 can lead to the activation of the NF-κB pathway and AP-1 pathway through association with TRAFs. Activation of these transcription factors leads to the expression of genes for cell survival. Thus TNFR1 has dual signaling capabilities for either cell death or cell survival. TNFR2 does not contain a death domain but does activate NF-κB. Thus, TNFR2 does not induce apoptosis but does lead to the activation of cell signaling events.


TNF-α
TNFR1
TNF-α
TNFR2
Apoptosis MAPK cascade
P
NIK
P
JNK
P
IKK
IκB
NF-κB
P
IκB
Degradation
AP-1
NF-κB
TNF Induced Gene Expression
Inflammatory proteins
Receptors
Chemokines
Adhesion molecules
Enzymes
Cytokines

Our studies suggest that reactivation is a multistep process which is initiated by factors induced as a result of allogeneic transplantation. On the basis of these results, we have hypothesized that TNF released by allogeneic stimulation activates transcription factors which regulate expression of the IE1 gene. Given the fact that IE 1 gene expression is very low or is not detectable in tissues from latently infected mice and that expression of this gene is required to initiate productive viral infection in vitro, induction of IE 1 gene expression is likely to be a key step in viral reactivation.

1.4. Goals of the Thesis

I would like to identify molecular mechanisms leading to transcriptional activation of IE gene expression in vivo. Since activation of IE gene expression may initiate or potentiate CMV reactivation, understanding the regulation of IE gene expression is crucial to understanding the mechanisms of reactivation. Previous studies in Abecassis lab and others suggested that TNF could be an important factor in inducing IE gene expression in allogeneic transplants [112]**.** To test this hypothesis, we used MIEP-lacZ transgenic mice. By using these mice, we can study the mechanism of regulation of activation of HCMV enhancer in response to allogeneic transplantation. Since these mice are not infected, increased expression of the reporter gene can only be due to activation of the enhancer, and not to lack of immune control in a naïve recipient. Allogeneic transplants were performed using MIEP-lacZ transgenic mice which could not respond to TNF due to deficiency in the TNF receptors (Kim, S.J. et al, manuscript in preparation). My studies demonstrate that TNF signaling contributes to activation of the HCMV IE enhancer in an allogeneic transplant. In addition, my studies demonstrate that ischemia-

reperfusion injury activates the enhancer independently of TNF receptor mediated signaling. We suggest that this likely occurs through activation of AP-1 and NF-κB induced by oxidative stress [113].

Although these findings await confirmation using animal models of viral infection, these studies may provide further insight into the molecular mechanisms by which reactivation of cytomegalovirus (CMV) from latency occurs. Furthermore, these studies suggest that new therapeutic approaches targeting the signaling pathways leading to activation of NF-κB as well as AP-1 may reduce the transcriptional activation of IE gene expression and, thus result in decreased reactivation of CMV in immuncompromised patients.

CHAPTER 2

Critical role for the TNF receptors in mediating activation of HCMV major immediate early enhancer in allogeneic transplantation

2.1. Introduction

Previously, our lab has found that allogeneic transplantation induced expression of the MCMV IE gene in donor kidneys from latently infected mice and of an MIEP-lacZ transgene in which the β-galactosidase gene is under the control of the HCMV IE promoter/enhancer [112]. Allogeneic transplantation induces expression of a complex array of genes involved in an inflammatory immune response, including cytokines which activate transcription factors, which in turn induce expression of additional genes involved in an inflammation. We wished to determine which of these factors could be important in inducing IE gene expression in the context of allogeneic transplantation. Our studies initially focused on the role of TNF because a number of lines of evidence suggested that TNF could play also a key role in reactivation of CMV [95-99, 107-110]. Based on previous data from our lab and others, we hypothesized that TNF, induced as a result of allogeneic transplantation, could be important in inducing IE gene expression in vivo. In order to investigate the requirement for TNF signaling in activation of the HCMV enhancer, we bred MIEP-lacZ mice to mice deficient in TNFR1, TNFR2 or in both TNFR1 and TNFR2. We first examined the response of these mice to soluble TNF to determine whether deficiency in TNFR1 and TNFR2 was sufficient to abrogate the response to TNF. We then examined the response of these mice to allogeneic transplantation. For these studies, kidneys from MIEP-lacZ TNFR +/+ or TNFR-deficient mice were used as donors for allogeneic transplantation. Cells in these kidneys would not

be able to respond to TNF. The activity of the HCMV MIEP/E was assessed by measuring the level of β-galactosidase activity in the transplanted kidney versus that of the contralateral control kidneys taken from the same donors (Fig. 7). We hypothesized that activation of the enhancer in allogeneic transplants occurs through activation of the transcription factors that control the MIEP/E. We therefore analyzed the activation of transcription factors that are thought to be important in regulation of MIEP/E.

In this chapter, I found that TNF signaling is an important pathway which contributes to activation of the HCMV MIEP/E in allogeneic transplantation. However, my studies suggest that TNF-independent pathways may also contribute to activation of the MIEP/E in this context. Chapter 3 explores TNF-independent pathways which lead to activation of HCMV enhancer.

2.2. Materials and Methods

2.2.1. Animals

Breeding pairs of MIEP-lacZ transgenic mice from the TG1JB line carrying a single copy of the β-galactosidase gene under the control of the HCMV MIEP/E were obtained from Jay Nelson at Oregon Health Sciences University [6]. These mice were bred with C57BL/6-*Tnfrsfla*^{tm1Imx} TNFR1 KO (H-2^{b}) mice or B6.129S2-*Tnfrsf1b*^{tm1Mwm} TNFR2 KO (H-2^{b}) mice (Jackson Lab) to generate either MIEP-lacZ TNFR1 KO or MIEP-lacZ TNFR2 KO mice respectively. MIEP-lacZ TNFR DKO mice were generated by breeding MIEP-lacZ TNFR1 KO mice with B6;129S-*Tnfrsfla*^{tm1Imx}*Tnfrsf1b*^{tm1Imx} mice (H-2^{b}) (Jackson Labs) (Fig. 6). C3H/HeSnJ (H-2^{k}) mice (Jackson Labs) were used as allogenic transplants recipients.

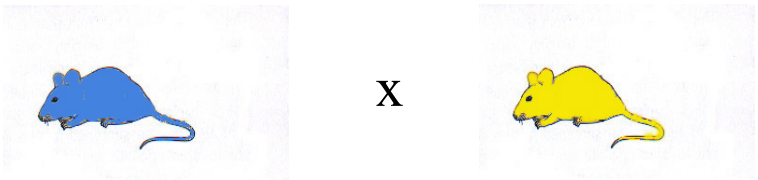
Mice were maintained in isolation cages and were fed and watered ad libitum. This study protocol was reviewed and approved by the Northwestern University Institutional Animal Care and Use Committee. Breeding was done by Gail Thomas.

2.2.2 Genotyping.

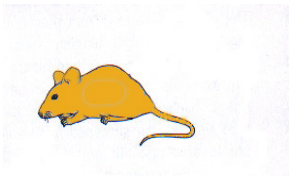
Genotyping for the presence of the transgene was done by Gail Thomas. DNA was purified from tail snips and 250 ng of DNA was analyzed for the presence of the *lacZ* gene with primers β-galactosidas1 (GCATCGAGCTGGGTAATAAGCGTTGGCAAT) and β-galactosidas2 (GACACCAGACCAACTGGTAATGGTAGCGAC) using cycling conditions A: 1 cycle 94 °C, 2 min, followed by 45 cycles of 94°C, 1 min, 55°C, 2 min, 72°C, 1.5 min, and 1 cycle of 72°C, 10 min. Genotyping for TNFR1 KO mice, TNFR2 KO mice, and TNFR DKO mice was done by Mary Hummel. DNAs were screened for the presence of the wild-type and mutant TNFR1 genes using primers p60B (GGATTGTCACGGTGCCGTTGAAG) and p60E (TGACAAGGACACGGTGTGTGGC) to amplify the wild type gene (120 bp) and primers p60-spe (TGCTGATGGGGATACATCCATC) and pgk5'-66 (CCGGTGGATGTGGAATGT GTG) to amplify the mutant gene (155 bp) with cycling conditions B: 94°C, 2 min, followed by 40 cycles of 94°C, 1 min, 65°C, 1 min, 72°C, 30 sec, and 1 cycle of 72°C., 10min. (Jackson Labs). DNAs were screened for the presence of the wild type TNFR2 gene with primers IMR 338 (CCTCTCATGCTGTCCCGGAAT) and IMR 339 (AGCTCCAGGCACAAGGGCGGG) (Jackson labs) (205 bp) using cycling conditions C: 94$_o$C. 2 min for 1 cycle; 94°C, 1 min, 60°C, 1 min, 72°C, 30sec for 40 cycles; 72°C. 10 min. for 1 cycle. The mutant TNFR2 gene present in the

Figure 6: Generation of MIEP-LacZ TNFR1 KO, TNFR2 KO, TNFR DKO mice. **(A)** MIEP-LacZ transgenic mice were bred with either TNFR1 KO mice or TNFR2 KO mice to generate MIEP-LacZ TNFR1 KO mice or MIEP-LacZ TNFR2 KO mice respectively. **(B)** MIEP-LacZ TNFR1 KO mice were crossbred with TNFR DKO mice. MIEP-LacZ TNFR DKO mice were obtained. The presence of transgene and genotype of each mice were performed using PCR by Gail Thomas and Dr. Hummel respectively.

A.

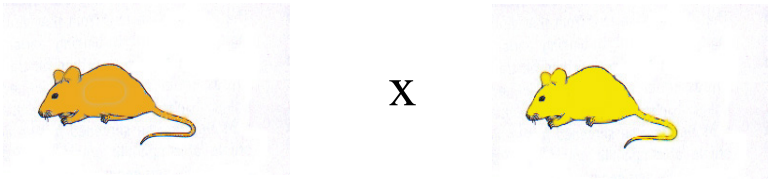


MIEP-LacZ Transgenic(+/+) X **TNFR1KO or TNFR2 KO**



MIEP-LacZ TNFR1 KO or MIEP-LacZ TNFR2 KO

B.



MIEP-LacZ TNFR1 KO X **TNFR DKO**

MIEP-LacZ TNFR DKO

B6;129S*Tnfrsf1a*$_{tm1Imx}$*Tnfrsf1b*$_{tm1Imx}$ mice was detected with primers p80-Kas (AGAGCTCCAGGCACAAGGGC) (Jackson labs) and pgk5'-66 (160 bp) using cycling conditions C.

2.2.3. **Transplants and organ processing**

Heterotopic mouse kidney transplants were performed as previously described by Jenny Zhang [114]. The left donor kidney was taken out at the time of transplantation as a control and stored at -80°C for later anlaysis. The right donor kidney from MIEP-lacZ WT, TNFR1 KO, TNFR2 KO mice, or TNFR DKO mice (H-2 $^{b/d}$) was transplanted into allogeneic C3H/HeSnJ (H-2^{k}) mice (Fig. 7). Except as noted, donor kidneys were removed 2 days after transplant. All organs were frozen in liquid nitrogen immediately after removal.

2.2.4 Cytokine injections

MIEP-lacZ WT, TNFR1 KO mice, TNFR2 KO mice, and TNFR DKO mice were injected intraperitoneally with 10μg of TNF (R&D Systems) or PBS and were sacrificed 24 h after injection for analysis of lacZ transgene expression. For gel shifts, mice were injected intraperitoneally with 10μg of TNF or PBS and were sacrificed after 2 h.

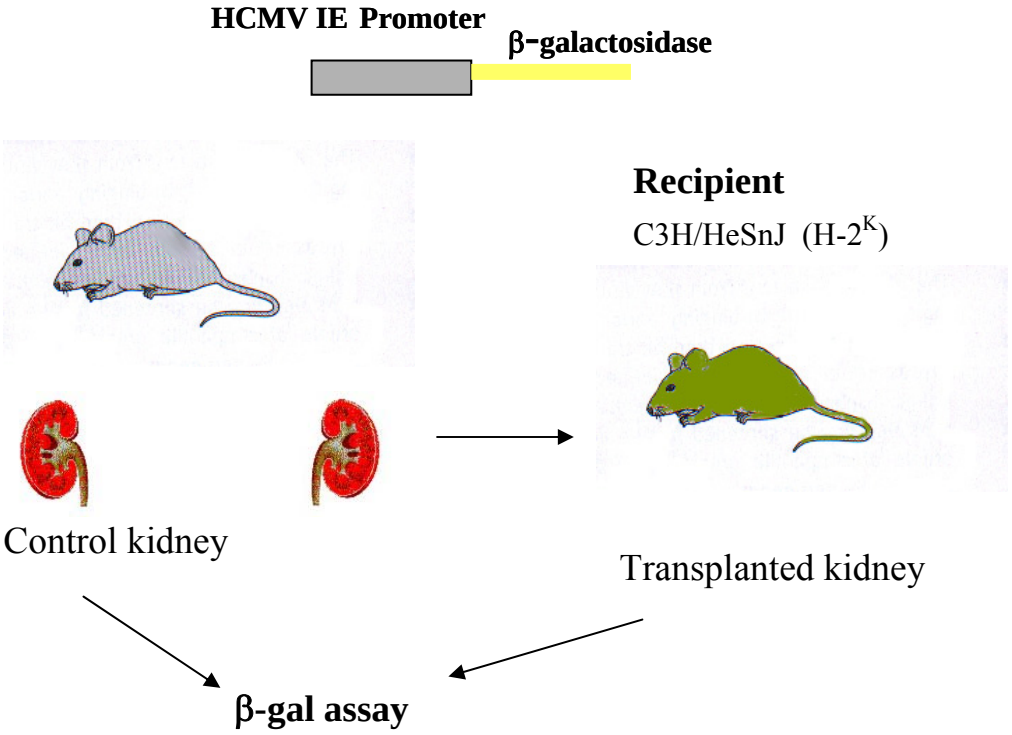
2.2.5 RT-PCR analysis.

For RNA extraction, frozen tissues were homogenized with a tissue tearer in TriReagent (Invitrogen) to disrupt the tissue and the RNA was purified according to the directions of the manufacturer. Reverse transcriptase PCRs (RT-PCRs) were performed with an RNA PCR kit (Perkin-Elmer Cetus) according to the directions of the manufacturer. RNA (1.5μg) was reverse transcribed using random hexamer primers, and the cDNA was amplified in 32 cycles of 94°C, 30s; 58°C, 30s; and 72°C, followed by a 7-min

Figure 7: Allogeneic transplantation of kidney from MIEP-lacZ transgenic mice into recipients. The right donor kidney from MIEP-lacZ WT mice, MIEP-lacZ TNFR1 KO mice, MIEP-lacZ TNFR2 KO mice, or MIEP-lacZ transgenic TNFR DKO mice (H-2 $^{b/d}$) which carry a β-galactosidase reporter gene under the control of the HCMV IE promoter/enhancer, were transplanted into allogeneic C3H/HeSnJ (H-2^{k}) mice and were removed 2 days after transplant. The left kidney was taken at the time of transplant and used as a control. All organs were frozen in liquid nitrogen immediately after removal. β-gal activity was analyzed as described in section 2.2.6.

Donor

MIEP-lacZ WT mice, MIEP-lacZ TNFR1 KO mice, MIEP-lacZ TNFR2 KO mice and MIEP-lacZ TNFR DKO (H-2 $^{b/d}$)



incubation at 72°C. PCR products were electrophoretically separated on 1% agarose gels. The primers used to amplify TNF were sens GAAAGCATGATCCGCGACGTGG antisens GTAGACCTGCCCGGACTCCGCAA, and generate 678bp [115] of RT-PCR products. The primers used to amplify β-actin were sens TGAGAGGGAAATCGTGCGTG and antisens ATCTGCTGGAAGGTGGACAGTGAG, and generate 453 bp of RT-PCR products.

2.2.6. **Analysis of lacZ transgenic expression**.

β-galactosidase activity in tissues from MIEP-lacZ transgenic mice was assayed with a Galacto-Star kit (Applied Biosystems). One half of each kidney was homogenized on ice in 750 µl of Galacto-Star lysis buffer containing 2µg/ml of PMSF and leupeptin with a dounce homogenizer. Once all the tissues were homogenized, tissue extracts were centrifuged at 4 °C, 12,000rpm for 5min. Supernatants were incubated at 48 °C for 60min in order to inactivate endogenous β-galactosidase activity. After heating, extracts were centrifuged at 4 °C, 12,000rpm for 5 min and supernatants were collected and stored in aliquots at -80°C.

For analysis of β-galactosidase activity, aliquots were diluted 1000-fold with lysis buffer and 10µl of diluted samples was incubated with 200 µl of reaction buffer containing galacton-star substrate and sapphire-II enhancer(1:50 ratio of galacto-star substrate to reaction buffer) for 1 hour at room temperature, at which time maximum light emission is reached. Light output was measured in triplicate with a Monolight 2010 luminometer.

Protein concentration in the extract was determined in duplicate by Bio-Rad protein assay, and values were expressed as average light units of β-galactosidase gene per nanogram of protein.

2.2.7. Preparation of nuclear extracts

Nuclear extracts were prepared from renal tissues with a Cellytic nuclear extraction kit (Sigma-Aldrich). Briefly, 100mg of renal tissues were treated with phosphatase inhibitor and homogenized with a dounce homogenizer on ice in 1ml ice-cold lysis buffer (10mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10mM KCl) with 10µl of 0.1 M DTT and 10 µl of the protease inhibitor cocktail (4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), Pepstatin A, Bestatin, Leupeptin, Aprotinin and trans-Epxoysuccinyl-L-leucyl-amido(4-guanidino)-butane). Homogenized tissues were centrifuged at 10,000 x g for 20 minutes at 4°C . The nuclear pellet was resuspended in 140 µl of extraction buffer (20mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol) containing DTT and protease inhibitor and gently agitated for 30 minutes at 4 °C. After centrifugation for 5 minutes at 20,000 x g at 4 °C, the supernatant was snap-frozen in aliquots with liquid nitrogen and stored at –70 °C. Nuclear extracts were assayed for protein concentration and used for both electrophoretic mobility shift assay and ELISA assay.

2.2.8. ELISA assay

Nuclear extracts were analyzed for activation of NF-κB, AP-1, and CREB with TransAM™ kits (Active Motif, Carlsbad, CA). Briefly, 5µg of nuclear extracts were added to 96-well plates to which oligonucleotide containing an NF-κB consensus binding site, a TPA-responsive element (TRE), or a cAMP-responsive element (CRE)

respectively had been immobilized. Samples were incubated for 1hour at room temperature with mild agitation (3 hours for CREB) and each well was washed 3 times with washing buffer. NF-κB p65 antibody, AP-1 antibodies (phopho-c-Jun, c-Fos, FosB, Fra-1, Fra-2, JunB or JunD antibodies), or phospho-CREB antibody was added and incubated for 1 hour at room temperature without agitation. After incubation, the wells were washed 3 times with washing buffer. HRP-conjugated antibody was added and plates were incubated for 1 hour at room temperature without agitation. The wells were washed 4 times with washing buffer. Developing solution (TMB substrate solution in 1% DMSO) was added to all wells and incubated 5 to 15 minutes at room temperature protected from direct light. The reaction was stopped by adding stop solution (0.5M H_2SO_4) and the absorbance was read on a spectrophotometer at 450nm.

2.2.9. Statistical analysis

T-test was used to determine statistical significance. Wilcoxon rank test was used to compare the fold induction in allogeneic transplants between control and TNFR DKO. P-values < 0.05 were considered significant.

2.3. Results

2.3.1. **Allogeneic transplantation induces MIEP-lacZ expression in transgenic mice at the peak of 2 days.**

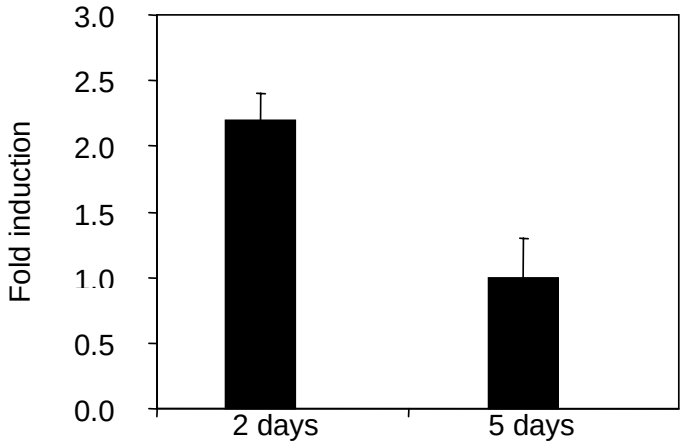
Our lab has previously shown that allogeneic transplantation resulted in induction of MCMV IE gene expression with a peak of 2 days posttransplant (POD). This activation of MCMV IE gene expression was variably detected at POD5 and not detectable at POD8. In order to investigate the kinetics of activation of the HCMV

enhancer in allogeneic transplantation, kidneys from MIEP-lacZ mice were transplanted into allogeneic C3H/HeSnJ ($H\text{-}2^k$) recipients. At POD2, the average β-galactosidase activity in transplanted kidneys was significantly higher than that of the contralateral controls (*p=0.001) (Fig. 12). The fold activation was determined by comparing β-galactosidase activity in the transplanted kidney versus that of the contralateral control for each mouse. The average activation at POD2 was 2.2-fold (range: 1.66-3.60, n=11) (Fig. 8). In contrast, no induction of MIEP-lacZ expression was observed at POD5. This demonstrates that expression of MIEP-lacZ was induced by allogeneic transplantation, with the peak of induction at POD2. At POD5, marked infiltration of CD8+ cells and destruction of the kidney architecture was observed in allogeneic transplanted kidneys. This suggests that the lack of activation of MIEP-lacZ expression at POD5 is due to the rapid allo-immune destruction of the transplanted kidney. Based on these data, MIEP-lacZ expression in subsequent studies was analyzed at 2 days after transplantation.

2.3.2. TNFR1 KO mice do not respond to soluble TNF

In order to investigate the response of MIEP-lacZ WT, R1KO, R2KO and DKO mice to TNF, either 10 μg of TNF or PBS was injected into MIEP-lacZ WT, TNFR1 KO, TNFR2 KO and TNFR DKO mice. MIEP-lacZ expression was analyzed at 24 hours after injection and the fold induction was calculated by comparing the average MIEP-lacZ expression between TNF injected mice and PBS injected mice. In WT mice and TNFR2 KO mice, TNF induced a statistically significant increase in MIEP-lacZ expression over PBS-treated controls (2.3-fold and 2.7-fold, respectively) (Fig. 9). In contrast, no induction of MIEP-lacZ expression was observed in MIEP-lacZ TNFR1 KO or MIEP-

Figure 8: Kinetics of activation of the MIEP/enhancer in allogeneic transplantation. Donor kidneys from MIEP-lacZ WT mice were transplanted into allogeneic recipients and removed 2 days and 5 days after transplant respectively. Contralateral donor kidneys were taken at the time of transplant and stored for later analysis as controls. Extracts from transplanted and control kidneys were analyzed in triplicate for expression of β–galactosidase activity and protein amount. β–galactosidase activity was calculated as light units per ng protein. The average fold induction in MIEP-lacZ expression was determined from the ratio of β-galactosidase activity in the transplanted kidney to that of the contralateral control kidney. Results are presented as fold induction + standard error. The average induction was 2.2-fold at 2 day after transplantation (range: 1.66-3.60, n=11) and was 1.0-fold (range: 0.28-2, n=5) at 5 day after transplantation.


3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fold induction
2 days
5 days

lacZ TNFR DKO mice (0.9-fold and 1.3-fold, respectively) (Fig. 9). These findings demonstrate that induction of MIEP-lacZ expression by soluble TNF occurs mainly through TNFR1. These results are consistent with previous studies, demonstrating that the effects of soluble TNF are mediated primarily by TNFR1 [116-119].

2.3.3. TNF-induced MIEP lacZ expression may occur through activation of NF-κB but not AP-1

Previous studies have shown that signaling through TNF receptors results in activation of both NF-κB and AP-1 [120, 121]. Our previous studies using gel shift assays showed that while TNF activates both NF-κB and AP-1 in the lungs of mice, TNF does not activate AP-1 in mouse kidneys [112]. I confirmed these results using a quantitative ELISA-based assay for NF-κB activation. This assay detects binding of the p65 NF-κB subunit to its cognate DNA sequence. The antibody recognizes an epitope on p65 which is accessible only when NF-κB is active and is bound to DNA. TNF activates the p65 component of NF-κB 17-fold in comparison to PBS injected controls at 2hr after injection (Fig. 10). In contrast, no activation of AP-1 was observed at the time after TNF injection (Fig. 10). This suggests that TNF activates MIEP-lacZ expression solely through activation of NF-κB. Additional studies using EMSAs to analyze transcription factor activation also showed that NF-κB, but not AP-1, was activated by TNF (data not shown).

2.3.4. **Expression of TNF is induced by allogeneic transplantation**.

Previous studies using MCMV latently infected Balb/c (H-2^d) mice, showed that expression of TNF is induced by transplantion into allogeneic C57BL/6 mice (H-2^b) [112].

Figure 9: Response of MIEP-lacZ mice to TNF. MIEP-lacZ WT (WT), MIEP-lacZ TNFR1 KO (R1KO), MIEP-lacZ TNFR2 KO (R2KO), MIEP-lacZ TNFR DKO (DKO) mice were injected intraperitoneally with either 10 μg of recombinant murine TNF or PBS and sacrificed 24 h after injection. Kidneys were harvested and analyzed in triplicate for β-galactosidase activity by chemiluminescence and for protein concentration as described in Methods and Materials. Data are presented as the average light unit (LU) per ng of protein plus standard error of the mean. MIEP-lacZ mice:PBS n=8, TNF n=9; R1KO mice: PBS n=6, TNF n=6; R2KO mice: PBS n=4, TNF n=4; DKO mice: PBS n=10, TNF n=11. TNF induced a statistically significant increase in β-gal activity in both MIEP-lacZ WT and MIEP-lacZ TNFR2 KO mice as compared to PBS-treated controls (*$p < 0.05$, t-test). In contrast, MIEP-lacZ TNFR1 KO mice and MIEP-lacZ TNFR DKO mice do not respond to TNF. β-gal activity is significantly higher in TNF-treated MIEP-lacZ WT mice than in TNF-treated MIEP-lacZ TNFR1 KO or MIEP-lacZ DKO mice (**$p < 0.05$, t-test).

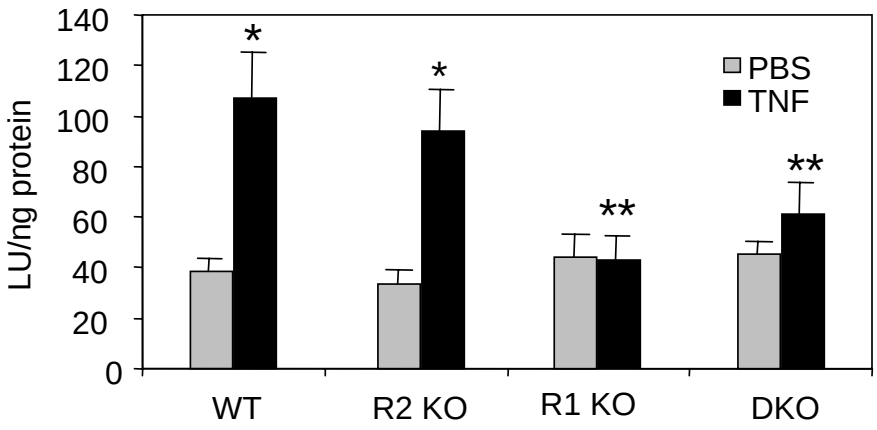
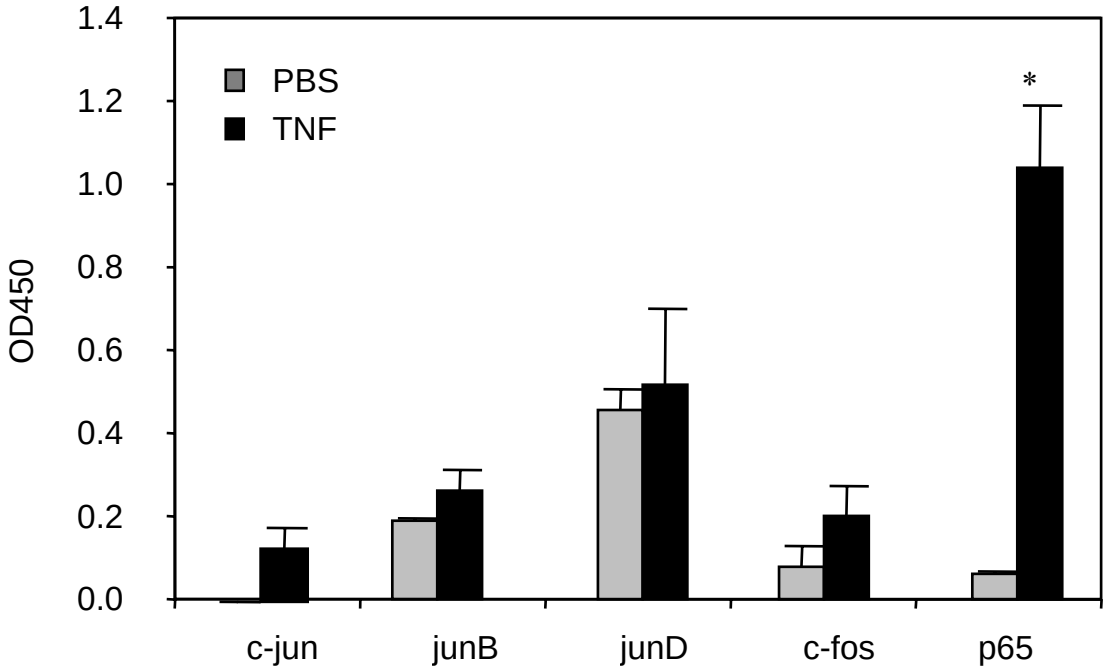

140
120
100
80
60
40
20
0
LU/ng protein
*
*
**
**
PBS
TNF
WT
R2 KO
R1 KO
DKO

Figure10: NF-κB activation in response to TNF. Mice were injected with TNF or PBS and then sacrificed 2 h later. Kidneys were harvested and nuclear extracts were prepared. Activation of the p65 component of NF-κB or various AP-1 family members was analyzed with a TransAM kit. TNF induced a statistically significant activation of NF-κB in kidneys. The fold activation of p65 (ratio in kidneys from mice injected with TNF vs. kidneys from mice injected PBS) was 17-fold. No activation of AP-1 family members was observed (*$p < 0.05$, t-test).


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD450
PBS
TNF
*
c-jun
junB
junD
c-fos
p65

To confirm that TNF expression is induced by transplantation of MIEP-lacZ mice into allogeneic C3H/HeSnJ (H-2^k) mice, I analyzed expression of TNF RNA. Little or no TNF expression was detected in control kidneys (Fig. 11, lane C). In contrast, expression of TNF was induced in transplanted kidneys (Fig. 11, lane G), demonstrating that TNF is specifically induced by allogeneic transplantation with this MHC mismatch.

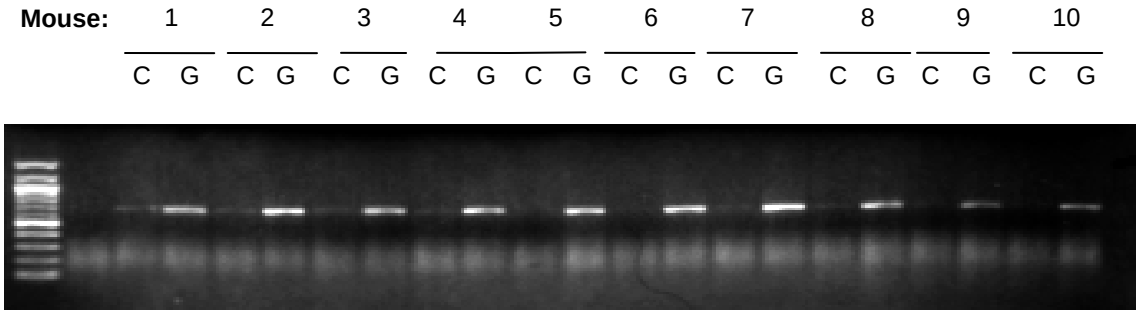
2.3.5 Signaling through TNFR2 contributes to induction of HCMV enhancer in an allogeneic transplant

In order to investigate the role of TNF signaling in the response of the MIEP to allogeneic transplantation, kidneys from MIEP-lacZ WT, MIEP-lacZ TNFR1 KO, MIEP-lacZ TNFR2 KO, or MIEP-lacZ TNFR DKO mice were transplanted into allogeneic recipients. Transplanted kidneys were removed at POD2 and MIEP-lacZ expression was measured as above. As with MIEP-lacZ WT mice, allogeneic transplantation of kidneys from MIEP-lacZ TNFR1 KO mice also induced a statistically significant increase in MIEP-lacZ expression ($p=0.03$), suggesting that genetic deficiency in the Type 1 TNF receptor had no effect on induction of MIEP-lacZ expression (Fig. 12A). While some increase in MIEP-lacZ expression was observed in transplanted kidneys from MIEP-lacZ TNFR2 or MIEP-lacZ TNFR DKO mice, the difference between control and transplanted kidneys was not statistically significant ($p=0.23$, and $p=0.07$, respectively). Variability in the level of β-galactosidase activity among the groups was observed in both control and transplanted kidneys. This might be due to experimental variation since kidneys from different transplants were analyzed over a long period of time. However, in each transplant, the control and transplanted kidneys were analyzed at the same time, and the

fold-activation was determined from the ratio of β-galactosidase expression in the transplanted kidney to that of the control. Calculation of the fold-activation removes inter-assay variation and therefore provides a better method for analysis of the data. There is a statistically significant difference in the fold-activation between WT and TNFR DKO or TNFR 2KO mice. MIEP-lacZ expression was 1.4-fold and 1.6-fold in MIEP-lacZ TNFR2 KO and MIEP-lacZ TNFR DKO mice, respectively (Fig.12B). The fold-activation in MIEP-lacZ TNFR2 KO and MIEP-lacZ TNFR DKO mice was significantly lower than that observed in MIEP-lacZ WT mice. These results demonstrate that signaling through the TNF receptors contributes significantly to activation of the HCMV IE enhancer in allogeneic transplantation, and that this occurs primarily through TNFR2.

Figure 11: Effect of transplantation on expression of TNF. The contralateral kidney was removed at the time of trnasplntation and used as a control (lanes C). Kidneys from MIEP-lacZ mice were transplanted (lanes G) into into allogeneic C3H/HeSnJ (H-2^k) mice and were removed 2 days after transplant. RNAs were analyzed for expression of TNF (A) as well as β-actin (B). RT-PCR products were detected by agarose gel electrophoresis. TNF expression was induced by allogeneic transplantation as shown in a previous study [112].

A. TNF-α



B. β-actin

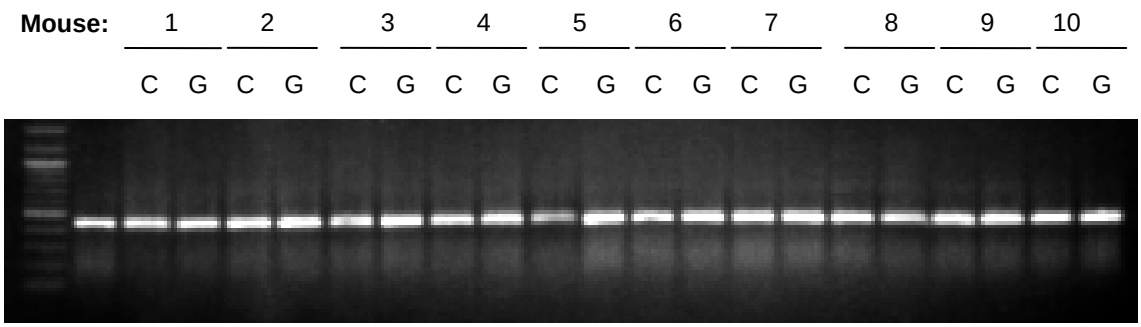
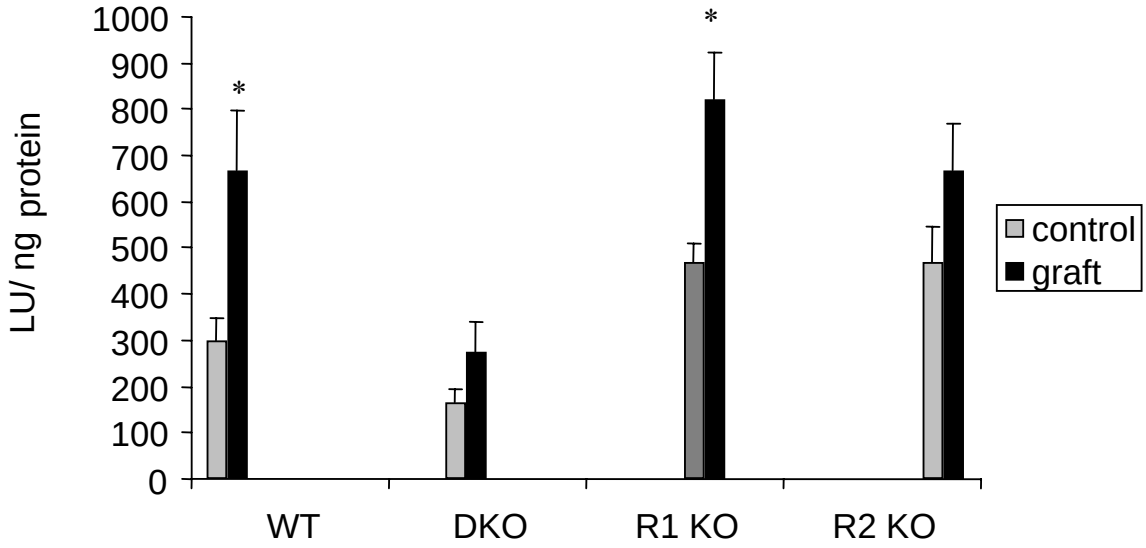


Figure 12: Analysis of the role of the TNF receptors in activation of the HCMV enhancer in allogeneic transplants. Donor kidneys from MIEP-lacZ WT (WT, n=11), MIEP-lacZ TNFR1 KO (R1 KO, n=6), MIEP-lacZ TNFR2 KO (R2 KO, n=8), or MIEP-lacZ TNFR DKO (DKO, n=8) mice were transplanted into allogeneic recipients C3H/HeSnJ mice and removed 2 days after transplant. Contralateral donor kidneys were removed at the time of transplant and stored for later analysis as controls. (A) Results are presented as average light units/ng protein plus standard error of the mean. Statistically significant increase in β-galactosidase activity were observed with MIEP-lacZ (WT) and MIEP-lacZ TNFR1 KO mice, but not with MIEP-lacZ TNFR2 or MIEP-lacZ DKO mice (*, $p<0.05$, t-test). (B) Fold induction was calculated as described in Fig 8. Results are presented as average induction plus standard error of the mean. Deficiency in TNFR2 or both in TNFR1 and TNFR2 results in a statistically significant decrease in the fold-induction of β-galactosidase activity (*, $p<0.05$, Wilcoxon rank test)

A.


1000
900
800
700
600
500
400
300
200
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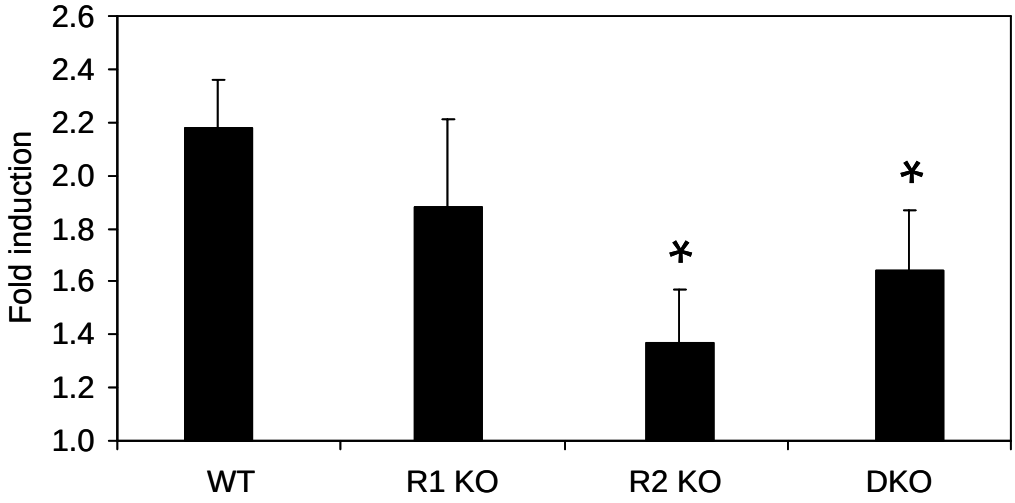
0
LU/ ng protein
*
*
WT
DKO
R1 KO
R2 KO
control
graft

B.


2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Fold induction
*
*
WT
R1 KO
R2 KO
DKO

Although not statistically significant, some increase in MIEP-lacZ expression in response to allogeneic transplantation, was detected in TNFR2 and TNFR DKO mice. This suggests that TNFR-independent signaling pathways may also contribute to induction of the gene in an allogeneic transplant.

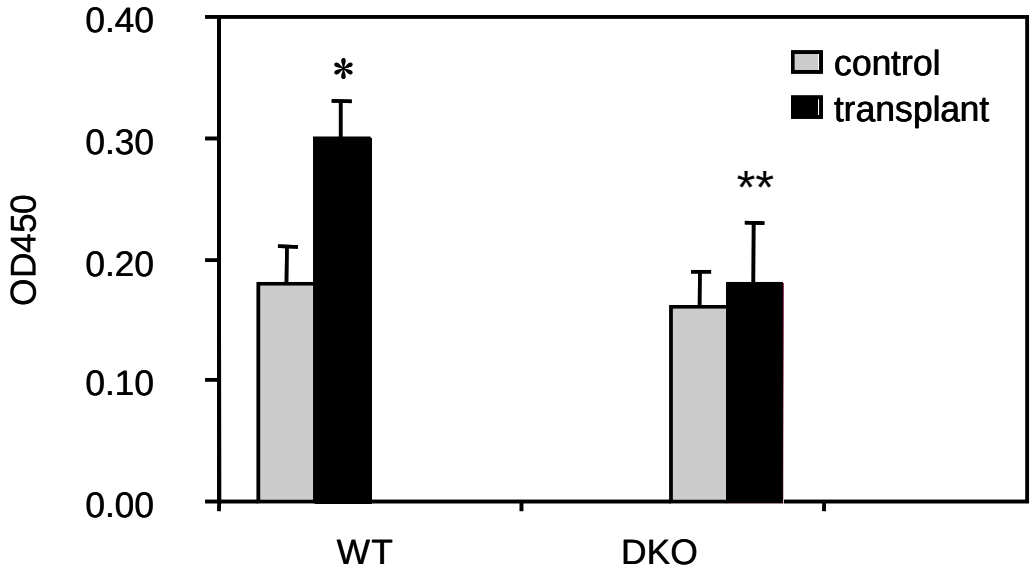
2.3.6. **NF-κB is activated by allogeneic transplantation**

In order to gain insight into signaling pathways which contribute to activation of the IE enhancer in allogeneic transplantation, we analyzed activation of transcription factors thought to be important in regulation of the enhancer. TNF activates NF-κB and HCMV enhancer regions have multiple binding sites for NF-κB. Furthermore, NF-κB sites have been shown to be important in regulation of HCMV promoter in many studies [12, 38, 43] and our lab showed that NF-κB is activated in allogeneic transplants [112]. We therefore investigated activation of the p65 component of NF-κB by a quantitative ELISA-based assay. NF-κB was significantly activated in transplanted kidneys from WT mice in comparison to control kidneys, but not in kidneys from TNFR DKO mice (Fig. 13). These results suggest that the reduced expression of MIEP-lacZ gene in TNFR DKO mice is likely due to failure to activate NF-κB.

2.3.7. AP-1 is activated by allogeneic transplantation independently of TNF

Our lab has shown by EMSA that allogeneic transplantation activates AP-1 at POD2. Previous studies have shown that AP-1 is activated by TNF and oxidative stress[122, 123]. Thus to determine whether AP-1 activation could contribute to induction of MIEP-lacZ expression in allogeneic transplants, activation of AP-1 family members was examined in

Figure 13: Effect of allogeneic transplantation on activation of NF-κB. In allogeneic transplants, the right donor kidney was removed for a control, and the left donor kidney from MIEP-lacZ TNFR WT (WT, n=5) mice or MIEP-lacZ TNFR DKO (DKO, n=4) mice were transplanted into allogeneic C3H/HeSnJ mice and removed at POD2 and stored at -80°C. Nuclear extracts were prepared and levels of p65 of NF-κB subunit in nuclear extracts from MIEP-lacZ TNFR WT and MIEP-lacZ TNFR DKO were analyzed quantitatively by ELISA. Allogeneic transplantation induced a statistically significant activation of NF-κB in kidneys from WT but not from DKO mice (* $p<0.05$, t-test). Activation of NF-κB in WT mice is significantly different from that in DKO mice (** $p<0.05$, t-test).


0.40
0.30
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OD450
*
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control
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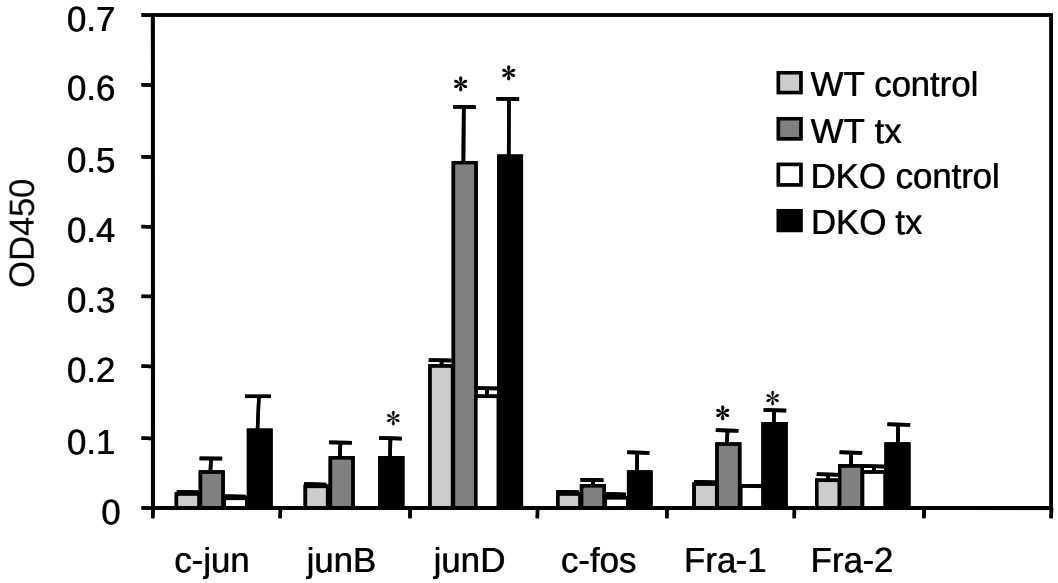
control and transplanted kidneys from MIEP-lacZ WT and MIEP-lacZ DKO mice. AP-1 is a family of transcription factor complexes. AP-1 complexes are composed of homodimers or heterodimers composed of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun dimerization partners (JDP1 and JDP2), or the activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) subfamilies. Strong activation of JunD was observed in allogeneic transplants of MIEP-lacZ WT mice (Fig. 14). Similar levels of activation of junD were also observed in transplants of MIEP-lacZ DKO mice, indicating that activation of JunD occurs independently of signaling through the TNF receptors. Weak, but significant activation of Fra-1 was also observed in both WT and DKO mice (Fig. 14). No activation of JunB, c-Fos, c-Jun, or Fra-2 was detectable at POD2 in allogeneic transplants of either WT or DKO mice (Fig. 14). Very weak but significant activation of junB was observed in DKO mice but not in WT mice (Fig. 14). This may reflect experimental variation rather than true activation. In any case, amount of activated JunB detected in DKO mice is very low, suggesting that it is unlikely to contribute to activation of the HCMV enhancer. In summary, AP-1 is activated by allogeneic transplantation in a TNF-independent manner.

2.3.8 CREB and Sp-1 are not activated in an allogeneic transplantation.

In the MIEP region, there are four CRE elements (TGACGTCA) and 3 TRE elements ($TGA^{G}/_{C}T^{C}/_{A}A$), which are potential binding sites for members of the CREB family. Members of AP-1 family can also bind to these sites since AP-1 and CREB/ATF can bind to the same sites and can heterodimerize [38-40, 124, 125]. The CREB/ATF family

Figure 14: Effect of allogeneic transplantation on activation of AP-1. The right donor kidney was removed for a control, and the left donor kidney from MIEP-lacZ TNFR WT (n=5) mice and MIEP-lacZ TNFR DKO (n=5) mice were transplanted into allogeneic mice. Nuclear extracts were prepared from kidneys from these mice. Activation of various AP-1 family members was analyzed quantitatively by ELISA at POD2. Activation of Fra-1 and JunD was observed in both TNFR WT (n=5) and TNFR DKO mice (n=5). Activation of junD was especially strong. (*$p<0.05$, t-test). Differences between WT and TNFR DKO mice in activation of other AP-1 family members were not statistically significant.


0.7
0.6
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0.4
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OD450
WT control
WT tx
DKO control
DKO tx
*
*
*
*
*
c-jun
junB
junD
c-fos
Fra-1
Fra-2

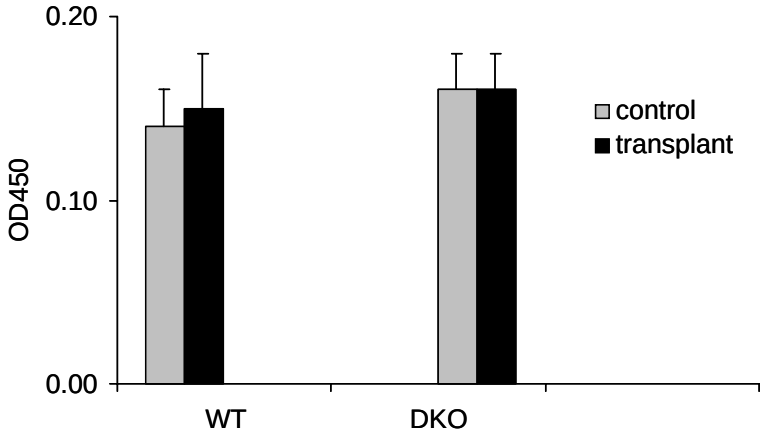
consists of a large number of transcription factors that include CREB, CREM, ATF-1, ATF-2, ATF-3, and ATF4 (also known as CREB2)[126, 127]. Because previous studies suggested that the CREB/ATF sites are important in regulation of the HCMV promoter, CREB-1 activation was analyzed in allogeneic transplantation by ELISA. CREB-1 was not activated at POD2 in transplants from either MIEP-lacZ WT or MIEP-lacZ DKO mice (Fig. 15A). The activity of other members of CREB/ATF family was not analyzed because the assay is specific for CREB-1. In addition to CRE sites in the MIEP region, there are three 21-bp repeats containing possible binding sites for YY1, Est.2 repressor factor, and Sp-1 sites. Levels of activated Sp-1 were significantly lower at POD2 in kidneys from both MIEP-lacZ WT and DKO as compared with controls (Fig. 15B).

2.4. Discussion

In this chapter I have investigated the role of TNF receptor signaling in activation of the HCMV enhancer in response to allogeneic transplantation. TNF is a primary mediator of immune regulation and the inflammatory response. There are two forms of TNF. TNF is first synthesized as a 26-kDa transmembrane protein. This transmembrane form of TNF is cleaved proteolytically by TACE to release a mature, soluble circulating protein of 17-kDa. Signaling by TNF is initiated by ligand-induced clustering of monomeric TNF receptors into dimers or trimers by TNF homotrimers. TNF signaling is mediated through two receptors, type1 (55kd) and type2 (75kd) [122]. Consistent with previous results in the Abecassis lab, I found that soluble TNF induces MIEP-lacZ expression. As expected, knocking out both receptors eliminated the response to TNF. While deficiency in TNFR1 completely abrogated induction of MIEP-lacZ expression in

Figure 15: Analysis of CREB-1 and SP-1 activation in POD2 allogeneic transplants. Nuclear extracts were prepared from MIEP-lacZ TNFR WT mice (n=5) and MIEP-lacZ TNFR DKO mice (n=5) were prepared. Levels of Sp-1 and CREB-1 in nuclear extracts of kidney from these mice were analyzed quantitatively by ELISA. Allogeneic transplantation down-regulate Sp-1 in both MIEP-lacZ TNFR WT and MIEP-lacZ TNFR DKO mice. (*$p < 0.05$, t-test). Fold activation (ratio in transplanted kidneys vs. control kidneys) was 0.48-fold and 0.44-fold in MIEP-lacZ TNFR WT and DKO mice, respectively. No activation of CREB was observed in both TNFR. Fold activation (ratio in transplanted kidneys vs. control kidneys) was 1.2-fold and 1.1-fold in MIEP-lacZ TNFR WT and DKO mice, respectively.

A. CREB-1


0.20
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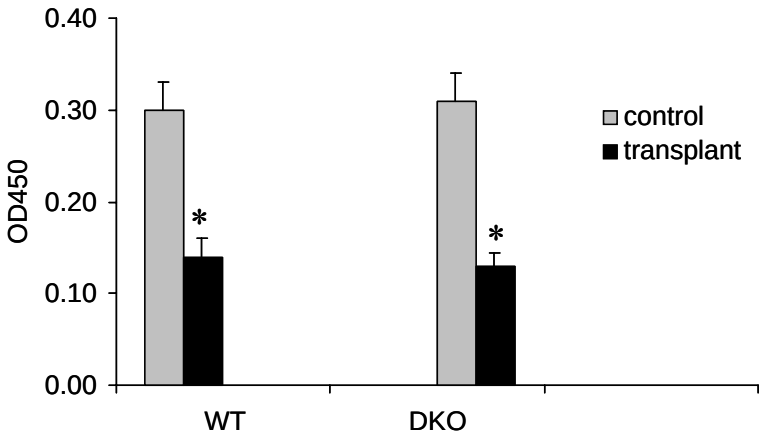
control
transplant
WT
DKO

B. Sp-1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0.30
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OD450
control
transplant
*
*
WT
DKO

response to TNF, deficiency in TNFR2 had no effect. Thus, signaling through TNFR1 mediates the response to soluble TNF.

My studies also demonstrated that allogeneic transplantation results in activation of the enhancer and that signaling through the TNF receptors plays a critical role in this model. Although TNFR1 mediated signaling activates the enhancer in response to soluble TNF, activation of the enhancer in allogeneic transplants was mediated primarily by TNFR2. While TNFR1 mediates signaling from both membrane-bound and soluble TNF, TNFR2 is preferentially activated by the membrane-bound TNF [128-130]. The TNF receptors are differentially expressed in the kidney in normal and disease states. TNFR1 is strongly expressed within the Golgi complex in the glomerular endothelium in normal kidneys [131]. This expression is lost in glomeruli from acutely rejecting human kidneys. In contrast, TNFR2 expression is not detectable in normal kidneys, but is upregulated in epithelial cells of the distal convoluted tubules (DCT) in acutely rejecting kidneys [131]. Consistent with its function and pattern of expression, TNFR1 alone could induce MIEP-lacZ expression in response to soluble TNF and MIEP-lacZ expression was limited primarily to the glomerulus in kidneys of TNF-injected mice [112]. Although there was a significant increase in MIEP-lacZ expression in allogeneic transplantation, the fold-increase in MIEP-lacZ expression in transplanted kidney was relatively low. This is likely due to the restricted expression of TNFR2 and the low frequency of cells responding to TNF. In summary, these studies suggest that TNFR1 in glomerulus is likely to mediate activation of HCMV enhancer in response to soluble TNF and TNFR2 in DCT is likely to be responsible for activation of HCMV enhancer in allogeneic transplantation. Further studies will be needed to demonstrate this conclusively.

Previous studies have shown that TNFR2 plays a central pathogenic role in other models, including concanavalin A induced hepatitis and glomerulonephritis[132, 133]. Our findings show that signaling via TNFR2 also contributes to activation of MIEP-lacZ expression in the local environment of inflammation induced by allogeneic transplantation.

Although not statistically significant, some activation of the enhancer was detectable in transplanted kidneys from TNFR-deficient mice. This suggests that TNFR-independent pathways may also contribute to activation of the MIEP/E in response to allogeneic transplantation. Allogeneic transplantation induces both an inflammatory immune response due to recognition of foreign antigens and oxidative stress due to ischemia/reperfusion (I/R) injury. The effect of I/R injury will be further investigated in the next chapter using a model of warm renal I/R injury.

Since activation of NF-κB is important in the transcriptional activation of gene expression in response to TNF, activation of NF-κB was examined in allogeneic transplants [120, 121]. Our results showed that although activation of NF-κB was observed in donor kidneys from WT mice, no activation of NF-κB was observed in kidneys from TNFR-deficient mice. These results suggest that the reduced activation of MIEP-lacZ expression in kidneys from TNFR DKO mice is due to failure to activate NF-κB. In order to study the requirement of NF-κB activation, I investigated the effect of several NF-κB inhibitors including a proteosome inhibitor, MG132 on activation of MIEP-lacZ expression in response to TNF. No inhibition of induction on MIEP-lacZ expression was observed. This may have been due to inappropriate dosage or timing of administration or

to instability of the compound in vivo. Therefore, the requirement for NF-κB in activation of the MIEP/E in response to allogeneic transplantation could not be tested.

In order to identify transcription factors other than NF-κB which might contribute to activation of the enhancer, we analyzed activation of other transcription factors with putative binding sites in the MIEP/E. In MIEP/E region, there are five 19-bp repeat elements containing consensus or near-consensus CREB/activating transcription factor (ATF) binding sites. Earlier studies suggested that these sites might be important in regulation of the HCMV promoter. CREB proteins are representatives of a class of ubiquitous and consititutively expressed transcription factors [134]. CREB can bind as homodimers or heterodimers to the cAMP response element (CRE). Activation of CREB by phosphorylation increases its DNA binding affinity to CREs. CREB-1 was not activated in our model and thus does not seem to play a role in activating HCMV enhancer in allogeneic transplants. Activation of other CREB family members was not analyzed. In addition to CREB sites, there are three 21-bp repeats containing consensus binding sequences for YY1, Est.2 repressor factor, and Sp-1. The potential contribution of Sp-1 in induction of MIEP-lacZ expression was evaluated. Sp-1 activity was lower in transplants than in controls. Although we cannot rule out the possibility that CREB-1 or Sp-1 may have been activated at earlier time points and subsequently inactivated, these results suggest that CREB and Sp-1 are unlikely to play a role in inducing MIEP-lacZ expression in allogeneic transplants.

Activation of AP-1 was also investigated in WT and TNFR DKO mice in allogeneic transplants. AP-1 is a family of transcription factor complexes composed of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB

and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) subfamilies [135, 136]. Additionally, some of the Maf proteins (v-Maf, c-Maf and Nrl) can heterodimerize with c-Jun or c-Fos, whereas other Maf related proteins, including MafB, MafF, MafG and MafK, heterodimerize with c-Fos. Jun proteins can form stable dimers that bind AP-1 DNA recognition elements (5'-TGAG/CTCA-3'), also known as TREs [phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements] [136]. AP-1 dimers can also bind to CRE elements. Studies have demonstrated that c-jun and c-fos positively regulate cell proliferation [137] and these are antagonized by JunB, a negative regulator of c-Jun induced cell proliferation [138, 139].

In our studies, dramatic activation of AP-1 was consistently observed in transplants. JunD and Fra-1 were significantly activated independently of TNF. JunD is protective against oxidative stress induced by hypoxia. Recent studies have shown that this protective effect is due to up-regulation of genes involved in the antioxidative response [140]. Our results suggest that activation of JunD and Fra-1 occurs largely as a result of oxidative damage due to I/R and may contribute to the TNF-independent activation of MIEP-lacZ expression observed in allogeneic transplants.

In this chapter, I have found that TNF signaling contributes to activation of the HCMV enhancer in response to allogeneic transplantation and that this activation is likely to occur primarily through activation of NF-κB. These data are consistent with the results of recent studies, suggesting that latency is maintained by transcriptional silencing of immediate early gene expression and that reactivation of latent virus begins through transcriptional activation of immediate early gene. Although not statistically significant,

some activation of HCMV enhancer was observed in TNFR-deficient mice, indicating other factors such as oxidative stress induced by I/R, may also contribute to activation of the enhancer in this model. In chapter 3, I examined the role of renal oxidative stress in activation of the enhancer, using a model of warm ischemia/reperfusion injury.

CHAPTER 3

Renal ischemia/reperfusion injury induces HCMV IE gene expression independently of TNF

3.1. Introduction

Allogeneic transplantation results in injury due to immune recognition of allo-antigens and nonspecific injury due to deprivation of oxygen resulting from interruption of blood flow (ischemic) and further injury caused by restoration of blood flow (reperfusion injury). A key component of early I/R is oxidative stress due to generation of reactive oxygen species (ROS) [141, 142].

In vitro studies demonstrated that ROS formed by CMV infection is crucial to IE gene expression, viral replication and virus-induced cytophathic effects [143-145]. I/R is known to activate AP-1 and NF-κB [146-148]. Based on these findings, we hypothesized that ROS activates signaling pathways leading to activation of transcription factors, including NF-κB and AP-1, which leads to activation of the HCMV enhancer. To test this hypothesis, we used a renal pedicle clamping model [149] with MIEP-lacZ mice to study the response of the HCMV MIEP/E to ischemia/reperfusion injury. In this model, kidneys were subjected to 45 minutes of ischemia by clamping the renal artery and vein. The clamp was then released, allowing blood to flow back into the kidney. MIEP-lacZ expression was compared between the kidney subjected to I/R and the contralateral kidney (Fig. 16). To investigate the role of TNF receptor signaling in activation of the MIEP in response to I/R, we investigated the effect of I/R on both WT MIEP-lacZ and TNFR-deficient MIEP-lacZ mice. In addition, activation of transcription factors that are thought to be important for regulation of the MIEP/E, was investigated. Finally, reactive

oxygen species (ROS), a key mediator of I/R injury, were measured in control and ischemic kidneys.

In this chapter, I found that I/R activates the CMV enhancer in kidneys of MIEP-lacZ mice. This occurs independently of signaling through the TNF receptors. My results suggest that activation of the enhancer may occur primarily as a result of ROS-mediated activation of AP-1. These studies have been recently published [113].

3.2. Materials and Methods

3.2.1. **Warm ischemia-reperfusion model and organ processing**

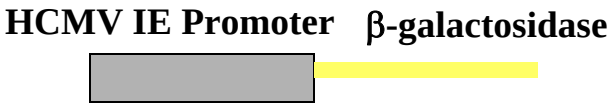
We utilized a well-established murine model of renal ischemia/reperfusion injury [149]. Briefly, the left renal pedicle was occluded with a nontraumatic vascular clamp for 45 minutes, during which time the kidney was kept warm and moist. The clamp was then removed and the kidney observed for return of blood flow. The mice were allowed to recover in a warmed cage after the peritoneum was sutured. After 0 min, 30min, 45min, 90min, 4hours, 7hours, or 24hours of reperfusion, the mice were sacrificed. The kidneys were snap-frozen in liquid nitrogen and stored at -70°C until further processing. The right kidney was used as a control (Fig. 16). Because previous studies have shown that I/R-induced release of TNF can affect the untreated contralateral kidney, control kidneys were removed prior to clamping the left kidney [150].

3.2.2. Electrophoretic mobility shift assay

NF-κB and AP-1 consensus oligonucleotides (Promega Co., Madison, Wis) were labeled with [γ-32] ATP (3,000 Ci/mmol, 10 mCi/ml; Amersham, Arlington Heights, Ill.) using T4 polynucleotide kinase. Extract (5μg) in 10μl was preincubated with 2 μl of gel shift

Figure 16: Warm ischemia/reperfusion model. MIEP-lacZ transgenic mice were anesthetized and the right kidney was removed as a control. The left kidney was subjected to 45 minutes of ischmia by clamping renal artery and vein, followed by various times of reperfusion.

MIEP-LacZ transgenic (H-2 $^{b/d}$)



Control kidney

45min ischemia, followed by reperfusion

binding buffer (20% glycerol, 5mM MgCl2, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl pH 7.5, 0.25mg/ml poly(dI-dC)·poly(dI-dC)) at 25°C for 10min and was then incubated with 1μl of probe for 20min at 25°C. The samples were loaded into 6% gels and the gel was run at room temperature in 0.5 X TBE buffer at 150V for 3 hours. The gel was placed on a sheet of Whatman filter paper after the gel plates were opened, covered with plastic wrap and dried on a gel dryer. The gel was exposed to X-ray film overnight at -70°C. Competition experiments were performed by incubation of extracts with a 100-fold excess of unlabeled oligonucleotide containing consensus or mutant NF-κB binding sites for 10 min prior to addition of the probe.

Oligonucleotide Sequence

NF-κB 5'-AGT TGA GGG GAC TTT CCC AGG C-3'

3'-TCA ACT CCC CTG AAA GGG TCC G-5'

AP-1 5'-CGC TTG ATG AGT CAG CCG GAA-3'

3'-GCG AAC TAC TCA GTC GGC CTT-5'

3.2.3. ELISA assay.

Activation of transcription factors was analyzed as described in chapter 2. The amount of NF-κB activated was determined using the standard curve with recombinant protein, p65 (Active Motif, Carlsbad, CA).

3.2.4. Lipid peroxidation assay.

10% (w/v) tissue extracts were prepared by homogenizing with a dounce homogenizer in a solution of 4mM butylated hydroxytoluene (BHT) and 10mM Tris, pH 7.4. These extracts are analyzed for the presence of 4-hydroxyalkenals (4-HNE) and

malondialdehyde (MDA) levels with a lipid peroxidation assay kit (Calbiochem, San Diego, CA).

In addition, the level of lipid hydroperoxides (LOOHs) was measured using a lipid peroxidation assay kit II. Tissue homogenates were centrifuged at 11,000 x g for one minute and the supernatant was used for the assay. 90 µl of the supernatant was treated with 10 µl of catalase to decompose the existing H_2O_2 in order to eliminate H_2O_2 interference from the LOOH measurement. To quantitate and subtract the non-LOOH contribution to the 560 nm signal, a sample blank was assayed after treatment with the reducing agent tris(2-carboxyethyl)phosphine (TCEP). The TCEP reduces lipid hydroperoxides to the corresponding organic alcohols, equation (TCEP + ROOH $\rightarrow$ TCEP=O + ROH). Therefore, same samples were treated with TCEP and without TCEP. The difference in absorbance at 560 nm for samples with and without TCEP is due to the LOOH content. The concentration of LOOH in the reaction mixture is calculated from the net absorbance using equation

$[LOOH]=(\text{Net } A_{560}/\varepsilon)\bullet\delta$

Where:

[LOOH]=Concentration of lipid Hydroperoxides in Sample (µM)

Net A_{560} = Net absorbance at 560 nm

$\varepsilon = 0.0431 \mu M^{-1}cm^{-1}$

δ = dilution factor = 11.2 (1.010 ml/ 0.090 ml)

Results were expressed as nmole/g tissue.

To assay MDA in combination with 4-HNE, 10% tissue homogenates were centrifuged at 3,000 x g for 10 minutes at 4°C and lipid peroxidation assay kit was used.

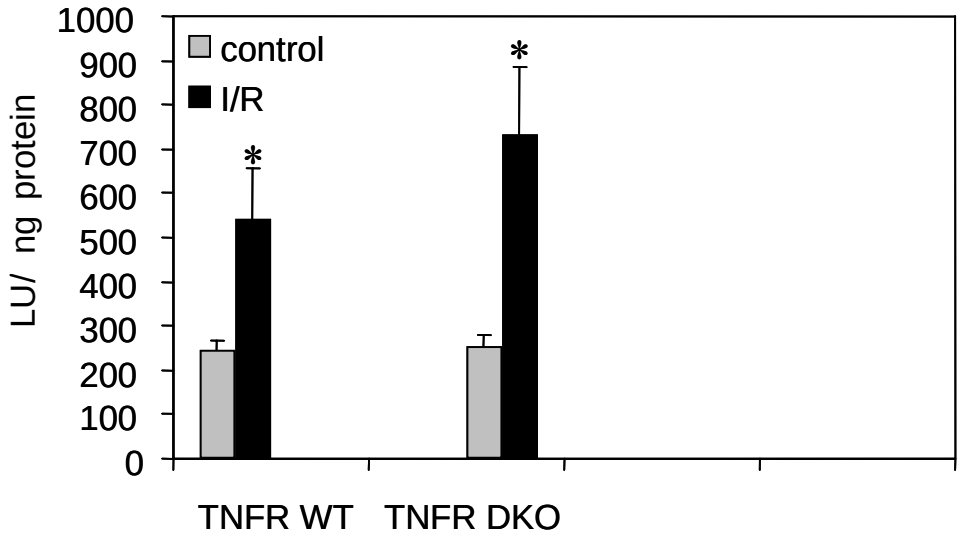
650µl of solution made of one volume of ferric ion/methanol and three volumes of N-methyl-2-phenylindole in acetonitrile was added to 200µl of collected supernatant. 150µl methanesulfonic acid was subsequently added and this mixture was incubated at 45°C for 60 minutes. Samples were cooled on ice and the absorbance of samples was measured at 586 nm. The concentration of MDA plus 4-HNE in the sample was calculated using a standard curve prepared from standards supplied by the manufacturer. The chromogenic reagent, N-methyl-2-phenylindole reacts with MDA and 4-HNE at 45°C. Condensation of one molecule of either MDA or 4-HNE with 2 molecules of N-methyl-2-phenylindole yields a stable chromophore with maximal absorbance at 586 nm.

3.3. Results

3.3.1. Renal ischemia/reperfusion injury induces MIEP-lacZ expression independently of TNF

I/R causes a series of responses, which results in release of several factors, including TNF and reactive oxygen species (ROS), which can activate NF-κB and AP-1 [150-153]. To investigate the effects of I/R injury on activation of the MIEP/E, we used a renal pedicle clamping model of warm ischemia and reperfusion injury on MIEP-lacZ mice (Fig. 16). I/R in wild type mice induced MIEP-lacZ expression approximately 2.3 fold in MIEP-lacZ mice subjected to 45min of ischemia followed by 24h of reperfusion (Fig. 17). No activation was detected at 90 min or 4 hr (data not shown). This induction is similar to that observed in transplants. To examine the role of TNFR mediated signaling in activation of MIEP-lacZ expression in response to I/R, I also analyzed the response of MIEP-lacZ TNFR DKO mice to I/R injury. I/R also induced activation of the enhancer in

Figure 17: Renal ischemia/reperfusion injury induces MIEP-lacZ expression independently of TNF. Kidneys from MIEP-lacZ WT (n=12) or MIEP-lacZ TNFR DKO mice (n=11) were subjected to 45 min of ischemia followed by 24 h of reperfusion. Kidneys were harvested and analyzed for β-galactosidase expression. The contralateral kidneys were removed prior to ischemia and used as controls. Data represents mean plus standard error. I/R injury induced a statistically significant increase in MIEP-lacZ expression in both MIEP-lacZ WT and MIEP-lacZ TNFR DKO mice ($P<0.05$, t-test). The average induction, calculated from the ratio of β-galactosidase activity in the kidney subjected to I/R versus the contralateral control kidney, was 2.4-fold (range: 0.71-5.63) in MIEP-lacZ WT mice and 3.1-fold (range: 0.74-7.65, $n=5$) in MIEP-lacZ TNFR DKO mice. The difference in induction between MIEP-lacZ WT and MIEP-lacZ TNFR DKO mice is not statistically significant ($P<0.05$, t-test).

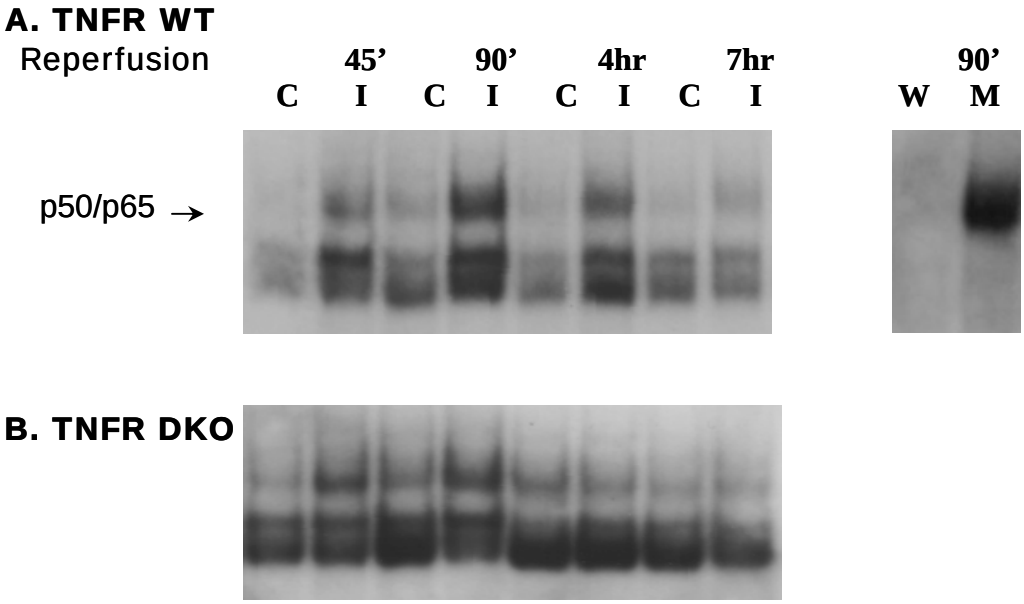

1000
900
800
700
600
500
400
300
200
100
0
LU/ ng protein
control
I/R
*
*
TNFR WT
TNFR DKO

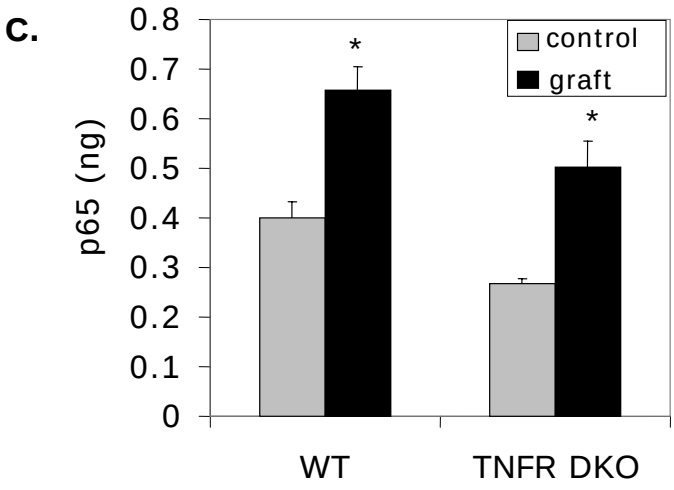
MIEP-lacZ TNFR DKO mice, and the extent of induction was not significantly different from that observed in MIEP-lacZ TNFR WT mice (Fig. 17). Thus, I/R injury induces transcriptional activation of the HCMV IE gene enhancer in vivo independently of signaling through the TNF receptors. Studies in the Abecassis lab have shown that I/R injury also induces MCMV IE gene expression in latently infected mice (Z.Li et al. unpublished observations).

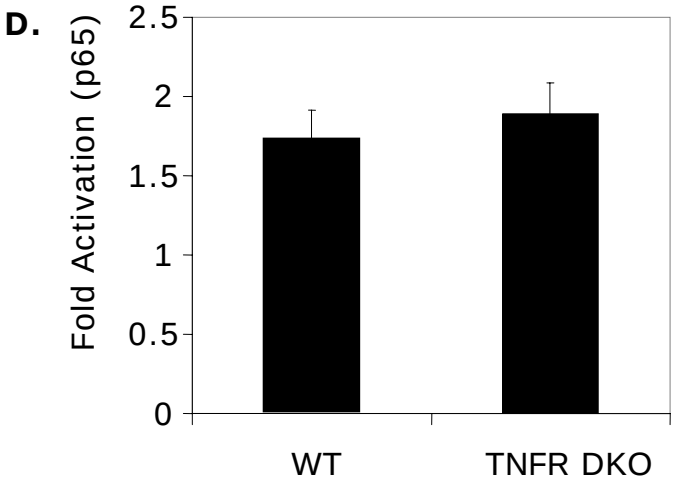
3.3.2 **NF-κB is activated by ischemia-reperfusion injury**

Because I/R injury has been shown to induce TNF expression and NF-κB activation [150-153], we examined the kinetics of NF-κB activation in response to I/R. Kidneys were subjected to 45 min of ischemia, followed by various times of reperfusion. Kidneys were harvested and analyzed for activation of NF-κB by gel shift assay and activation of the p65 NF-κB subunit was analyzed quantitatively using an ELISA-based assay for measuring binding to an NF-κB consensus sequence. Activation of NF-κB in MIEP-lacZ WT mice was first observed after 30 min of reperfusion and peaked at 90 min (Fig. 18A). No activation was observed at 4 h post-reperfusion (Fig. 18A). Quantitative analysis of p65 showed that NF-κB is activated approximately 1.7-fold at the peak time following I/R injury (Fig. 18D). Although the amount of activated NF-κB is significantly lower both in control and I/R injured kidneys of MIEP-lacZ TNF DKO mice than in WT mice, the kinetics and the fold-activation of NF-κB were similar between MIEP-lacZ TNF DKO mice and MIEP-lacZ TNFR WT mice (Fig. 18B, C). Activation of MIEP-lacZ expression was not observed until more than 4hr of reperfusion (data not shown). Thus, these results show that 1) NF-κB activation is weakly and transiently induced by ischemia/reperfusion

Figure 18: Effect of ischemia/reperfusion injury on activation of NF-κB. The right kidney from each mouse was taken as a control prior to ischemia (lanes C). The left kidney was subjected to 45 min of ischemia followed by 30 min, 45 min, 4 h, or 7 h of reperfusion (lanes I). Kidneys were harvested and analyzed for activation of NF-κB in MIEP-lacZ WT mice (A) or MIEP-lacZ TNFR DKO mice (B) by mobility shift assay. Samples from WT and DKO mice were analyzed simultaneously and exposed to film for the same length of time. Results presented are representative of a minimum of 2 samples at each time point. (C) The amount of p65 NF-κB subunit (ng) in nuclear extracts from MIEP-lacZ TNFR WT (n=9) and MIEP-lacZ TNFR DKO mice (n=7) were analyzed quantitatively by ELISA at 90 min post-reperfusion. The amount of p65 is lower in control and ischemic kidneys from MIEP-lacZ TNFR DKO mice than MIEP-lacZ WT mice. (D) Results in (D) are presented as the average fold activation calculated from the ratio of p65 in the ischemic kidney versus the contralateral control + standard error. Ischemia/reperfusion injury induced 1.7-fold activation of p65 (range: 1.2-5.2) in WT mice and 1.9-fold activation of p65 (range: 1.6-5.9) in TNFR DKO mice. The difference is not statistically significant (t-test, *$P<0.05$).


A. TNFR WT
Reperfusion
45'
90'
4hr
7hr
90'
C I C I C I C I W M
p50/p65
B. TNFR DKO


C.
p65 (ng)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
control
graft
*
*
WT
TNFR DKO


D.
Fold Activation (p65)
2.5
2
1.5
1
0.5
0
WT
TNFR DKO

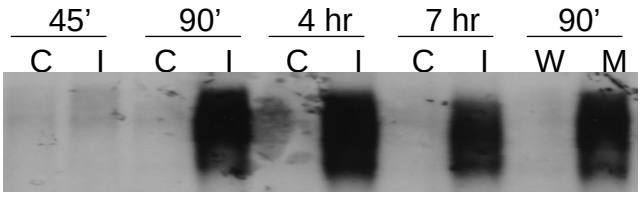
injury; 2) this activation occurs independently of signaling through the TNF receptors; 3) activation of NF-κB occurs prior to induction of MIEP-lacZ expression.

3.3.3. AP-1 is activated by ischemia-reperfusion injury independently of TNF and may be responsible for HCMV IE gene expression

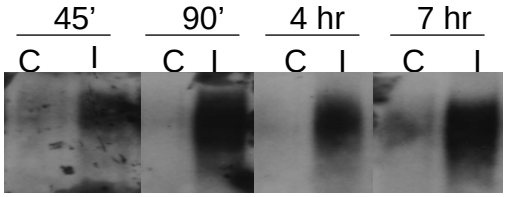
Renal I/R injury has been shown to activate AP-1 [151]. To determine whether AP-1 activation by I/R injury may contribute to induction of MIEP-lacZ expression, AP-1 activation in response to I/R injury was examined in MIEP-lacZ WT and MIEP-lacZ DKO mice. In contrast to NF-κB, AP-1 was strongly activated and activation was sustained for several hours (Fig. 19A). The kinetics and extent of AP-1 activation were similar between MIEP-lacZ WT and TNFR DKO mice (Fig. 19A, B), indicating that activation of AP-1 is TNFR-independent. AP-1 appeared to be heterogeneous by the gel shift assay. AP-1 is not a single protein but a collection of hetero or homo dimeric basic–leucine zipper (bZIP) proteins that belong to the Jun, Fos, Maf, and ATF subfamilies. Thus, the heterogeneity that I observed may be due to formation of different complexes.Therefore, activation of individual members of the AP-1 family was further investigated using a quantitative ELISA assay for measuring binding to an AP-1 consensus DNA sequence. Activation of AP-1 family members was analyzed at 4hr post-reperfusion, the peak time of AP-1 activation in gel shifts. Activation of c-Fos, c-Jun, JunD, and JunB was observed (Fig. 19B, C). No activation of FosB, Fra-1 or Fra-2 was observed at this time (data not shown). c-fos showed the greatest fold-increase in activation by I/R injury (Fig. 19D). The highest levels of activation were observed with JunD (Fig. 19C). No difference in the levels of activation of AP-1 family members between MIEP-lacZ WT and TNFR DKO mice was observed (Fig. 19 A, B, C, D).

Figure 19: Effect of ischemia-reperfusion injury on activation of AP-1. Control (lanes C) and ischemic (lanes I) kidneys from MIEP-lacZ WT mice were treated as in Fig. 18 and analyzed for activation of AP-1 in MIEP-lacZ WT mice (A) or MIEP-lacZ TNFR DKO mice (B) by mobility shift assay. Results are representative of a minimum of two samples at each time point. W, competition assay with 100-fold molar excess of unlabeled AP-1 oligonucleotide incubated with extract prepared from kidneys subjected to 45 min of ischemia and 90 min of reperfusion; M, competition assay with 100-fold molar excess of mutant AP-1 oligonucleotide incubated with the same extract. (C). Activation of various AP-1 family members was analyzed quantitatively using an ELISA-based assay at 4 h post-reperfusion. Differences in activation between WT (n=6) and TNFR DKO (n=6) mice are not statistically significant (t-test, $p>0.05$). (D) Results in (C) are presented as the fold activation of AP-1 family members calculated from the ratio in the ischemic kidneys versus the contralateral control + standard error (t-test, $*p<0.05$). c-fos was undetectable in control kidneys and was arbitrarily assigned a value of 0.01.

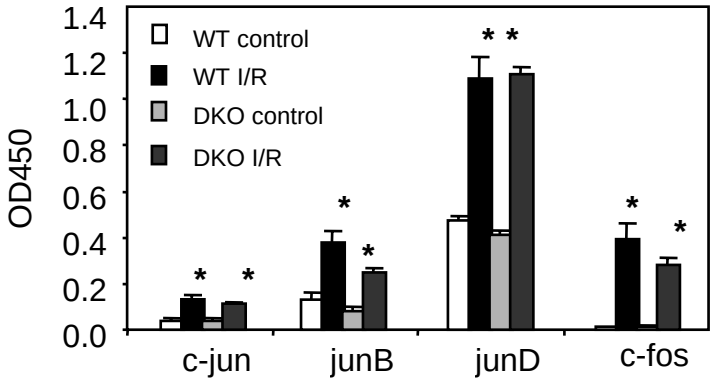
A. TNFR+/+



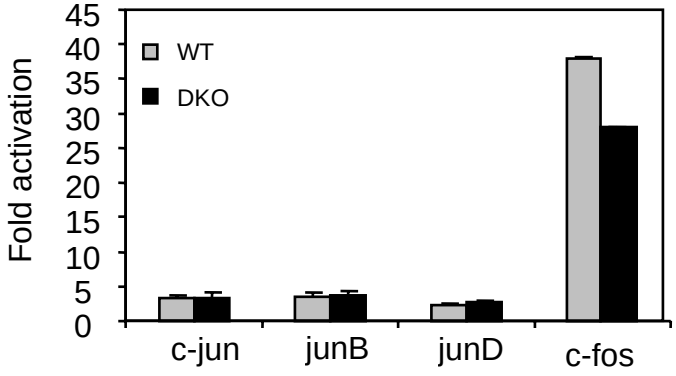
B. TNFR DKO



C.



D.



MIEP-lacZ was not expressed at 4h post-reperfusion, indicating that AP-1 activation precedes the induction of MIEP-lacZ expression in both MIEP-lacZ WT and TNFR DKO mice. In summary, 1) multiple AP-1 family members are activated by I/R injury; 2) activation of AP-1 occurred independently of signaling through the TNF receptors; 3) activation of AP-1 occurred prior to induction of MIEP-lacZ expression; 4) activation of AP-1 was strong, and in contrast to NF-κB, was sustained for several hours.

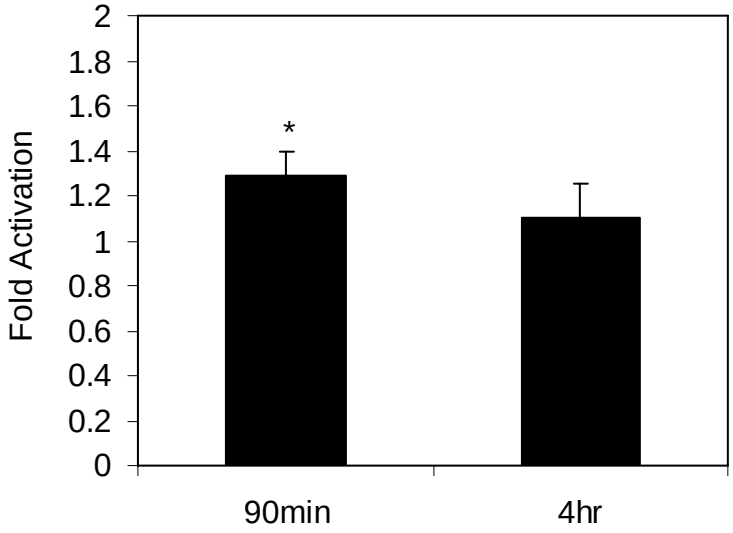
3.3.4 CREB is weakly activated by ischemia-reperfusion injury

Previous studies suggested that CREB/ATF sites may be important in regulation of the HCMV promoter. In a preliminary study, CREB-1 activation was examined in response to I/R injury by an ELISA-based assay at 30min, 45min, 90min, 4hours, or 7hours post-reperfusion in MIEP-lacZ WT mice. Small peaks of activation were observed at 90min and 4h post-reperfusion. Then, larger numbers of MIEP-lacZ WT mice were examined for CREB-1 activation at 90min or 4h post-reperfusion. A weak activation of CREB-1 was observed (1.3 fold; range: 0.85-1.65; n=9) at 90-min post-reperfusion but not later time points (Fig. 20). Thus, CREB-1 activation was very weak, transient and is unlikely to play a role in inducing MIEP-lacZ expression in response to I/R. Other members of the CREB family were not analyzed.

3.3.5. Renal ischemia/reperfusion injury induces reactive oxygen species (ROS) independently of TNF

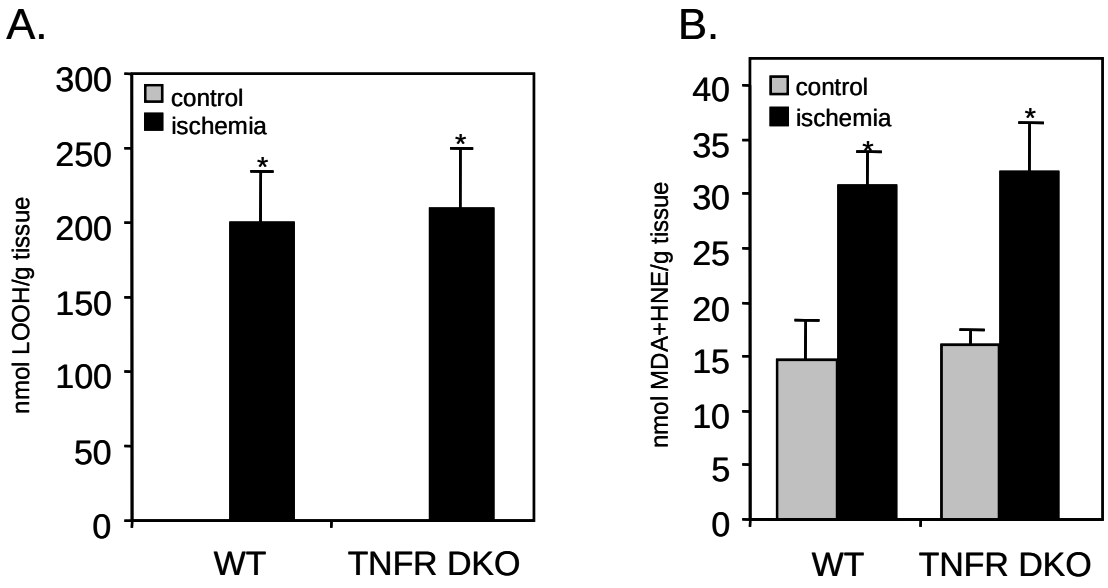
Previous studies have shown that multiple forms of reactive oxygen and nitrogen free radicals are generated during the oxidative stress associated with I/R injury. Thus results

Figure 20: Effect of ischemia-reperfusion injury on activation of CREB. The right kidney was taken as a control from MIEP-lacZ WT mice. The left kidney was subjected to 45 min of ischemia, followed by 90min and 4hours of reperfusion. The kidneys were analyzed for activation of CREB-1 at 90min (n=9) or 4h (n=5) post-reperfusion using an ELISA-based assay. Results are presented as the fold activation of CREB-1 calculated from the ratio in the ischemic kidneys versus the contralateral control + standard error (t-test, *$p<0.05$). Small, but significant activation of CREB-1 was observed at 90min post-reperfusion. However, no activation was detectable at 4h post-reperfusion.


Fold Activation
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
90min
4hr

in damage to all major cellular components, including membrane lipids, protein, carbohydrates, and DNA [141, 142]. The pathophysiological consequences are widespread tissue damage and associated inflammation. Several cellular signaling pathways are activated in response to ROS. The most significant effects of oxidants on signaling pathways have been observed in the mitogen-activated protein (MAP) kinase/AP-1 and NF-κB pathways [151-153]. We therefore analyzed the formation of reactive oxygen species in kidneys subjected to ischemia/reperfusion injury. Free radical species are extremely reactive and therefore short-lived, which makes reliable, and direct in vivo detection difficult. We therefore measured generation of reactive species indirectly by measuring 4-hydroxyalkenals (4-HNE) and malondialdehyde (MDA) formed as a result of lipid peroxidation of polyunsaturated fatty acids and esters. A 2-fold increase in 4-HNE and MDA was observed after 45 min ischemia (Fig. 21B). No increase in 4-HNE and MDA was observed after varing periods of reperfusion. In addition, we measured formation of lipid hydroperoxides. Lipid hydroperoxides (LOOH) were undetectable in control kidneys, but high levels were detected in ischemic kidneys (Fig. 21A). Although LOOH were detected immediately after ischemia, LOOH were not detected at any time following reperfusion, suggesting that they are turned over rapidly. No difference in ROS byproducts was observed between MIEP-lacZ WT and TNFR DKO mice. These results indicate that ROS are produced independently of TNF prior to activation of NF-κB and AP-1 and induction of MIEP-lacZ expression. Our results suggest that formation of ROS in response to I/R injury results in activation of transcription factors, which activate the enhancer of HCMV.

Figure 21: Effect of ischemia-reperfusion injury on generation of ROS. The right kidney was taken as a control. The left kidney from MIEP-lacZ (n=4) and MIEP-lacZ DKO mice (n=4) were subjected to 45 minutes of ischemia without reperfusion. Kidneys were analyzed for production of lipid hydroperoxides (A) or for 4-hydroxyalkenals (4-HNE) or malondialdehyde (MDA) (B). Ischemia significantly increases in LOOH levels and in 4-HNE+MDA levels in both WT and TNFR DKO (*$p<0.05$, t-test).


A.
300
250
200
150
100
50
0
control
ischemia
nmol LOOH/g tissue
*
*
WT
TNFR DKO
B.
40
35
30
25
20
15
10
5
0
control
ischemia
nmol MDA+HNE/g tissue
*
*
WT
TNFR DKO

3.4. Discussion

In this chapter I found that I/R injury induces transcriptional activation of the HCMV major IE promoter/enhancer in the kidneys of MIEP-lacZ mice. Previous studies have shown that expression of TNF RNA and protein is rapidly induced by renal I/R, but this induction is gone by 45 min of reperfusion. Consistent with these results, I did not observe TNF expression in kidneys subjected to 45 min of ischemia. Therefore, TNF message might be expressed at earlier time than 45 min of ischemia and degraded by this time. Earlier time points were not examined. Although previous studies have demonstrated that renal I/R induces TNF expression [150, 152], my studies demonstrates that activation of the enhancer occurs independently of TNFR signaling. Transcription factor activation and ROS production preceded activation of MIEP-lacZ gene expression. Therefore, the kinetics of these events is consistent with the hypothesis that I/R induces a burst of ROS production that activates transcription factors known to be important to regulate HCMV IE promoter/enhancer and this results in activation of MIEP-lacZ expression.

I/R injury is mediated initially by intracellular damage due to interruption of the blood supply (ischemia) and subsequent re-oxygenation due to restoration of blood supply (reperfusion). In organ transplantation, this complex chain of events leads to inflammation, production of ROS, and cell death by apoptosis and necrosis, organ damage and primary graft dysfunction. ROS are generated under normal, nonstressed physiological conditions. The toxic effects of ROS are prevented by superoxide dismutase and glutathione peroxidase, which convert superoxide to hydrogen peroxide, and subsequently to water. These natural defenses are overwhelmed during I/R injury [154].

ROS are generated primarily by mitochondria [142]. During I/R, ROS production increases because of leakage of electrons from the damaged electron transport chain, depletion of glutathione peroxidase, and reduction of superoxide dismutase activity. Furthermore, damage to the electron transport chain impairs ATP production and causes release of apoptogenic factors such as cytochrome c and cell death due to apoptosis or necrosis.

ROS and other free radicals such as NO influence the expression of a number of genes. Signal transduction pathways activated by ROS include cAMP mediated cascades, calcium-calmodulin pathways, and MAPK pathways. ROS are also known to activate transcription factors, including AP-1 and NF-κB in response to I/R [123].

Activation of NF-κB occurs in response to a number of inflammatory mediators including TNF, LPS, IL-1 and LTα through well-characterized signaling pathways. Although the mechanism is unclear, activation of NF-κB is also induced by ROS [152]. In our studies, we observed transient activation of NF-κB in response to I/R. The activation is not impaired in TNFR-deficient mice and is therefore likely due to TNFR-independent generation of ROS in response to injury. Given the weak and transient nature of the response, activation of NF-κB may not contribute significantly to activation of MIEP-lacZ gene expression in this model.

Growth factors, oncoproteins, such as v-Src or Ha-Ras, proinflammatory cytokines, such as TNF and IL-1, UV radiation as well as oxidative stress induce AP-1 activity, resulting in cell proliferation, neoplastic transformation and apoptosis. AP-1 activity induced by growth factors is likely to be mediated by the ERK MAP kinase (MAPK) cascade, whereas the responses to proinflammatory cytokines, UV radiation, and oxidative stress are mostly dependent on two other MAPK cascades, JNK and p38

[123, 155]. In contrast to NF-κB, activation of AP-1 in response to I/R was strong and was sustained for several hours. This suggests that activation of AP-1 is likely to be important in mediating activation of MIEP-lacZ expression. Activation of c-Jun, JunB, JunD, c-Fos was observed. JunD was strongly activated by both allogeneic transplantation and by I/R injury. A characteristic of JunD is its high basal expression in many cell types in the kidney. JunD can homodimerize or heterodimerize with other proteins including Fra-1, ATFa, ATF-3, Oct-1, CHOP (C/EBP homologous protein 10), a transcription factor induced by cellular stress [156, 157]. Interaction between JunD and CHOP has been shown to activate expression of AP-1 target genes [158]. Because of its long half-life, JunD may stabilize its partners, protecting them from degradation [134]. Previous findings showed that JunD homodimers weakly bind to AP-1 and CRE sites, but dimerization of JunD with Fra-1 dramatically enhances its binding to AP-1 and CRE sites [134]. As previously mentioned in chapter 2, JunD is protective against oxidative stress induced by hypoxia by up-regulating genes involved in the antioxidative response. Thus, JunD may be particularly important in activation of enhancer of HCMV in response to ischemic injury.

Recently, it has been reported in several studies that antioxidants such as aspirin or NAC, which inhibit formation of ROS, can inhibit IE gene expression and viral replication in vitro [43, 144, 159]. These studies support the hypothesis that ROS are important in regulation of HCMV IE gene expression and thus may contribute to activation of IE gene expression in vivo.

In this chapter, I have found that I/R-induced MIEP-lacZ expression occurred through TNF independent mechanisms. This induction was associated with strong and persistent activation of AP-1 and transient activation of NF-κB. Although we cannot rule

out the possibility that NF-κB or other factors may be important, our results suggest that AP-1 activation might be sufficient to induce transcriptional activation of MIEP-lacZ expression in response to I/R.

CHAPTER 4

CONCLUSIONS

The purpose of this thesis is to understand the mechanisms underlying activation of IE gene expression in vivo in response to surgical injury and allogeneic transplantation. Given that IE transcripts from the MIE region are the most abundantly expressed α genes of HCMV and are required for activation of viral gene expression in productive infection, induction of IE gene expression is likely to be the first step in reactivation of latent HCMV. Previous studies suggested that TNF-mediated activation of NF-κB could be an important pathway in inducing reactivation of the virus [111, 112]. It is known that TNF is sufficient to induce transcriptional reactivation of MCMV IE gene expression in latently infected mice and to induce expression of the MIEP-lacZ reporter gene under the control of the HCMV major immediate early promoter/enhancer [112]. Using these mice, I investigated the requirement for TNF signaling in allogeneic transplant-mediated induction of MIEP-lacZ expression. I found that TNFR signaling plays a critical role in induction of MIEP-lacZ expression in allogeneic transplants. Interestingly, my results show that this induction of MIEP-lacZ expression in allogeneic transplantation occurs mainly through TNFR2. Although not statistically significant, some induction of MIEP-lacZ expression was observed in TNF-receptor deficient mice. This TNFR-independent MIEP-lacZ expression in allogeneic transplantation may occur as a result of oxidative stress. I further investigated the role of oxidative stress in activation of MIEP-lacZ expression by using a model of renal ischemia-reperfusion injury.

Renal ischemia/reperfusion injury also induced MIEP-lacZ expression in both TNFR+/+ and TNFR-deficient mice. This induction was preceded by formation of reactive oxygen species (ROS) and activation of NF-κB and AP-1 in a TNFR-independent manner.

Taken together, these results suggest that there may be at least two pathways which contribute to induction of HCMV IE gene expression. TNF injection is sufficient to induce MIEP-lacZ expression and this is likely to occur primarily through activation of NF-κB. In allogeneic transplantation, TNFR-mediated signaling through activation of NF-κB contributes to activation of the HCMV enhancer. Alternatively, activation of the enhancer in response to I/R occurs through TNF-independent pathway which may be mediated primarily by activation of AP-1. Each pathway may be independent and sufficient to induce IE gene expression (Fig. 22). The observation that transient transfection of either NF-κB or c-Jun is sufficient to activate the enhancer but that they can act cooperatively to activate the human cytomegalovirus immediate-early gene enhancer/promoter in vitro, further supports the hypothesis [37]. Both pathways are likely to contribute to activation of IE gene expression in the context of human allogeneic transplantation since prolonged storage and transport times increase the extent of I/R injury in clinical settings.

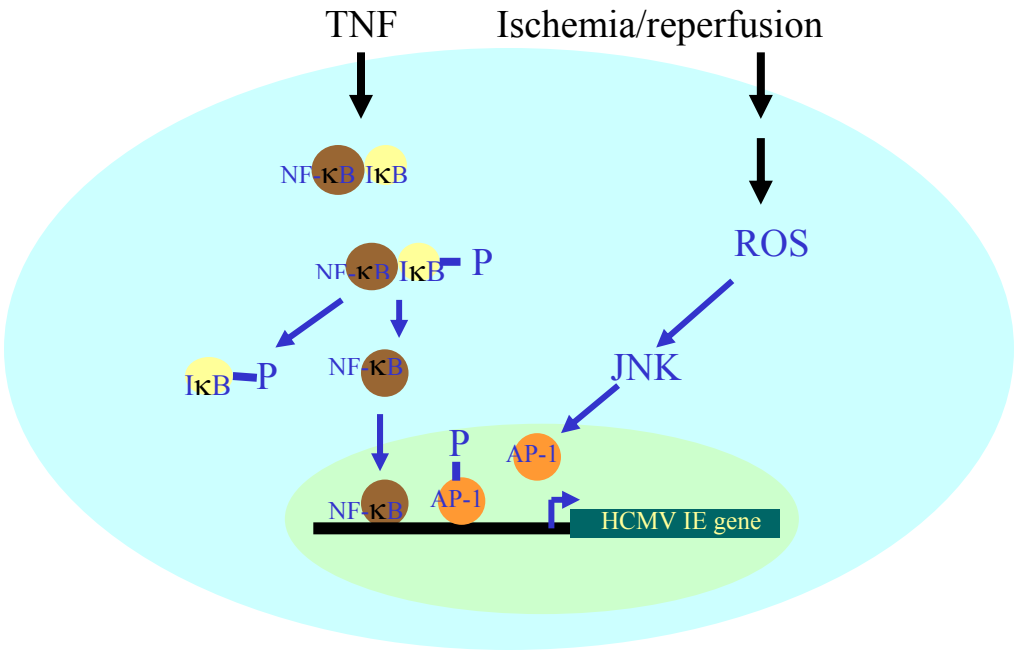
This thesis elucidates mechanisms involved in inducing activation of the HCMV enhancer in vivo and specifically shows the importance of TNF and oxidative stress in this process. High levels of HCMV-specific CD8+ T cells are detectable in seropositive individuals, suggesting that there is a high frequency of reactivation occurring [61-65]. We propose that infection or cell damage may also lead to activation of these pathways in

unmanipulated hosts, resulting in asymptomatic reactivation which is controlled by the host immune response.

Recent studies of reactivation from latent HCMV virus have focused primarily on undefined pathways of cellular differentiation as the trigger for reactivation. Although it will be important to validate these studies using in vivo models of viral infection, my studies suggest that an inflammatory immune response and activation of NF-κB and AP-1 may contribute to activation of IE gene expression and lead to subsequent reactivation of virus in immunocompromised allotransplant recipients.

Thus, my studies provide a different perspective as to how reactivation of latent virus may occur and suggest that targeting pathways that lead to activation of NF-κB and AP-1 may be of therapeutic value in patients to prevent reactivation of CMV.

Figure 22: Proposed mechanism for induction of IE gene expression in allotransplants. We propose that reactivation of CMV occur in transplant recipients as a result of TNF signaling which induces activation of NFkB, or as a result of activation of AP-1 by reactive oxygen species formed in response to ischemia/reperfusion injury. This leads to transcriptional activation of the *ie* genes, and, in immunocompromised hosts, to production of infectious virus.


TNF
Ischemia/reperfusion
NF-κB IκB
ROS
NF-κB IκB P
IκB P
NF-κB
JNK
P
AP-1
NF-κB
AP-1
HCMV IE gene

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