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Role of alveolar epithelial Early growth response-1 (Egr-1) in CD8⁺ T Cell mediated Lung Injury

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Abstract

Influenza infection of the distal airways results in severe lung injury, a considerable portion of which is immunopathologic and attributable to the host responses. We have used a mouse model to specifically investigate the role of antiviral CD8⁺ T cells in this injury, and have found that the critical effector molecule is TNF- α expressed by the T cells upon antigen recognition. Interestingly, the immunopathology which ensues is characterized by significant accumulation of host inflammatory cells, recruited by chemokines expressed by the target alveolar epithelial cells. In this study we analyzed the mechanisms involved in the induction of epithelial chemokine expression triggered by antigen-specific CD8⁺ T cell recognition, and demonstrate that the Early growth response-1 (Egr-1) transcription factor is rapidly induced in epithelial cells, both in vitro and ex vivo, and that this is a critical regulator of a host of inflammatory chemokines. Genetic deficiency of Egr-1 significantly abrogates both the chemokine expression and the immunopathologic injury associated with T cell recognition, and it directly regulates transcriptional activity of a model CXC chemokine, MIP-2. We further demonstrate that Egr-1 induction is triggered by TNF- α – dependent ERK activation, and inhibition of this pathway ablates Egr-1 expression. These findings suggest that Egr-1 may represent an important target in mitigating the immunopathology of severe influenza infection.

Keywords

Tumor necrosis factor (TNF- α); Interferon-gamma (IFN- γ); CD8⁺ T Cells; Influenza; Early growth response-1 (Egr-1); Chemokines; Lung injury

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Disclosure

The authors have no financial conflict of interest.

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Introduction

Significant lung injury frequently accompanies clinical and experimental respiratory virus infection, which may be mediated both by the direct effects of the virus as well as a result of the host immune response (La Gruta et al., 2007; Kash et al., 2006; Bruder et al., 2006). CD8⁺ T cells play an important role in host defense against respiratory viral infections such as influenza virus (Harty et al., 2000; Kohlmeier and Woodland, 2009). CD8⁺ T cell receptor (TCR) recognition of viral antigens in the form of small peptides bound to class I major histocompatibility (MHC) complex on the surface of lung epithelial cells, leading to the formation of an immunological synapse (Dustin, 2009). This critical event activates signal transduction pathways in both T cells and target antigen presenting cells (APC). TCR engagement triggers release of cytotoxic molecules such as perforin and granzyme as well as antiviral cytokines including interferon gamma (IFN- γ) and tumor necrosis factor (TNF- α) from the T cells (Slifka et al., 1999; Russell and Ley, 2002). We have previously shown that transcriptional activation and expression of inflammatory mediators by target cells in response to CD8⁺ T cell recognition (Ramana et al., 2006).

We have developed a transgenic mouse model for the purpose of dissecting the direct effects of CD8⁺ T cell recognition of viral antigen presented by alveolar epithelial cells, in the absence of replicating virus infection. The model antigen is the A/Japan/57 influenza Hemagglutinin (HA), expressed under the transcriptional control of the surfactant protein C (SP-C) gene promoter, resulting in alveolar epithelial expression (Enelow et al., 1996; Enelow et al., 1998). Adoptive transfer of cloned HA-specific CD8⁺ cytotoxic T lymphocytes (CTLs) leads to progressive lethal injury over a period of 3–6 days. The animals developed a progressive interstitial pneumonitis characterized initially by lymphocytic infiltration of alveolar walls and spaces, followed by an exuberant neutrophil and macrophage cell infiltration (Enelow et al., 1996). This model has been used to study the role of antigen-specific T cell recognition of an alveolar epithelial antigen and the resulting inflammatory cascade in the immunopathology of acute lung injury (Zhao et al., 2000; Small et al., 2001). Gene expression profiling studies revealed that CD8⁺ T cell recognition triggered the induction of TNF- α -dependent and IFN- γ -dependent genes in epithelial target cells (Ramana et al., 2006). We have shown in this model that lung injury is largely mediated by chemokines expressed by the epithelial cells upon T cell recognition mediated by TNF- α resulting in massive host inflammatory cell infiltration (Xu et al., 2004). TNF- α is a pleotropic cytokine that mediates a multitude of inflammatory events. Signaling by TNF- α is mediated by a variety of kinases and transcription factors, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERK1 and ERK2), p38, I-Kappa B Kinases (IKK) and members of activator protein (AP1), nuclear factor kappaB (NF- κ B), as well as Egr-1 which controls multiple aspects of inflammatory gene expression (Baud and Karin, 2001; Ghosh and Karin, 2002; Cao et al., 1992). Egr-1 represents a family of immediate-early response genes that contain a conserved zinc finger DNA-binding domain and bind to a GC-rich sequence in the promoters of target genes (Thiel and Cibelli, 2002; Silverman and Collins, 1999). Egr-1 has been shown to play an important role in multiple forms of lung injury by regulating inflammatory gene expression and may be involved in a variety of lung diseases including emphysema and pulmonary fibrosis (Zhang et al., 2000; Lee et al., 2004). However, signal transduction pathways involved in CD8⁺ T cell mediated activation of Egr-1 in lung epithelial cells remain to be established.

In this study, we have identified Egr-1 transcription factor as a potential mediator of inflammatory gene expression in MLE-Kd lung epithelial cells after CD8⁺ T cell recognition, as a result of the expression pattern observed in microarray analysis of epithelial gene expression. We have also demonstrated that CD8⁺ T cell recognition activates ERK kinases and induces significant increase in the level of Egr-1 expression, as well as CXC chemokine production in epithelial cells *in vitro* and *in vivo*. Prevention of Egr-1 induction by inhibition

of ERK activation abrogated macrophage inflammatory protein (MIP-2) production *in vitro* and genetic deficiency of Egr-1 results in dramatic abrogation of CD8⁺ T cell mediated lung injury. Promoter analysis and chromatin immunoprecipitation demonstrated that Egr-1 binds to a specific element in MIP-2 gene and mediates induction by TNF- α the primary mediator of CD8⁺ T cell induction of epithelial chemokine expression. Array analysis of *ex vivo* epithelial transcriptional activities revealed that Egr-1 regulates a large number of chemokines involved in neutrophil and macrophage influx that mediate inflammation resulting in severe lung injury, suggesting a critical role of target cell Egr-1 induction in the immunopathology associated with viral antigen recognition by T cells in the distal lung.

2. Materials and methods

2.1. Reagents

Mouse TNF- α and IFN- γ were purchased from Genzyme (Boston, MA) and PBL (New Brunswick, NJ), respectively. Antibodies for Egr-1, SP1 and NF- κ B (p65) were purchased from Santa Cruz Biotechnology (Santacruz, CA). Antibodies specific for dual phosphorylated-ERK (Thr204, Tyr204) and total ERK were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Santa Cruz biotechnology. Mek1/2 inhibitor U0126 was purchased from Cell Signaling Technology (Beverly, MA). Control (scrambled) and Egr-1 Si-RNA were purchased from Dharmacon, Inc (Chicago, IL). Egr-1 si-RNA was made by Dharmacon, Inc as a custom SMARTpool containing four different siRNA that has been experimentally validated by the manufacturer to block Egr-1 expression.

2.2. Animals

Homozygous Wild-type Egr1 (+/+) and Egr-1 Knock-out (-/-) mice on C57BL/6 background were generated by breeding heterozygous Egr1 +/- mice (Lee et al., 1996). These mice were purchased from Taconic farms (Hudson, NY). Wild-type and Egr-1 KO mice expressing the A/Japan/57 influenza hemagglutinin (HA) under the transcriptional control of the surfactant protein C (SP-C) promoter were generated by selective breeding of C57BL/6 with Balb/c mice expressing HA. All genotypes were confirmed by PCR. All experiments were conducted in strict accordance with the guidelines of the National Institute of Health (N.I.H) and Institutional Animal Care and Use Committee (IACUC).

2.3. T-lymphocyte culture and adoptive transfer

Wild type and Egr-1 KO HA+ transgenic mice were used at 12–16 week of age. Wild-type CD8⁺ T cell clone (40-2) specific for the 210–219 epitope of A/Japan/57 HA were used in adoptive transfer experiments (Xu et al., 2004). CD8⁺ T cells (5×10^6) were injected via the tail vein into appropriate recipients. Lungs were harvested at appropriate times for histology or type II alveolar epithelial cell preparation, as described (Xu et al., 2004).

2.4. Cell culture and cytokine treatment

Mouse lung epithelial (MLE-Kd) cells were maintained in DMEM supplemented with 10% FBS and antibiotics, penicillin and streptomycin (Ramana et al., 2006). Cells were treated with TNF- α (20ng/ml). Cells were pre-treated with indicated concentration of ERK inhibitor, U0126 (50 μ M) for 2 hrs prior to the experiment. In cell co-culture experiments, CD8⁺ T cells and MLE-Kd cells loaded with 10^{-9} M synthetic peptide representing the 210–219 epitope of the A/Japan/57 hemagglutinin (HA) were used at 2:1, effector: target (E/T) ratio. TNF- α -/- (TKO) CD8⁺ T cells were described previously (Xu et al., 2004; Ramana et al., 2006).

2.5. Gene expression profiling by microarrays

After co-culture, MLE-Kd and CD8⁺ T cells were separated using mouse anti-CD8⁺ dynal magnetic beads. The purity of separation was confirmed by Fluorescence Activated Cell Sorting (FACS). Total RNA was prepared from cells by using the RNeasy kit (Qiagen, CA). The integrity and quantity of RNA was verified on agarose-formaldehyde gel. 10 µg of total RNA was annealed to the oligo-dT-T7 promoter at 70°C for 10 min and then reverse transcribed at 42°C for 3 hrs. complementary RNA (cRNA) was synthesized using the mega-script kit from GIBCO/BRL (Frederick, MD) and biotinylated nucleotides. The labeled probes were purified and hybridized to the murine genomic array as directed by manufacturer's instructions (Affymetrix, CA). The washed arrays were stained with phycoerythrin-streptavidin (Molecular Probes, CA) and read using an Affymetrix Gene-chip scanner and analyzed using Gene Sifter software (Seattle, WA). Mouse chemokine arrays were purchased from Superarray, Inc (Frederick, MD). RNA was prepared from mouse lung type II cells of wild-type and Egr1-KO HA+ transgenic mice, 6h after CD8⁺ T cell transfer. Labeled probes were prepared and hybridized to chemokine arrays as recommended by the manufacturer. The intensity of the signals on the films were quantitated using NIH image software. The ratio of wild type and Egr-1 KO intensities was calculated and expressed as fold suppression in Egr1-KO mice. Gene array experiments were performed with three pooled RNA samples for each group (cells or mice). The array experiments were performed twice and data from a representative experiment was shown.

2.6. Enzyme linked immunosorbent assay (ELISA)

Cell culture supernatants were assayed for MIP-2 using a sandwich ELISA from BD PharMingen (San Diego, CA), according to the manufacturer's instructions. The experiment was performed independently three times, with comparable results. Standard errors are indicated.

2.7. mRNA Expression Analysis

RNA was extracted from cell pellets using the RNeasy kit. Reverse transcriptase-polymerase chain reactions (RT-PCR) were performed using Ambion Retroscript (Austin, TX), according to the manufacturer's protocol. Primer sequences for Egr-1, MIP-2, MCP-1, GAPDH and β -Actin were described previously (Lee et al., 1996; Jaruga et al., 2003). PCR products were resolved on a 1% agarose gel containing ethidium bromide and visualized with U.V. Light.

2.8. Western blot

Cell extracts were prepared and proteins were separated by electrophoresis using 8% – 10% SDS-PAGE gels. Proteins in the gel were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, CA), and subjected to immunoblotting with the indicated antibodies. Blots were visualized by enhanced chemiluminescence western detection system (Pierce, IL).

2.9. Luciferase and chromatin immuno precipitation (CHIP) assays

MLE-Kd cells were split into 6-well plates 1 day before the transfection at a concentration of 5×10^5 cells per well. Cells were transfected with wild type MIP-2 (–187 to +14) gene promoter linked to luciferase designated as pMIP-2(–187)wt-luc or with constructs with mutation in the NF- κ B or SP1/Egr-1 elements (Kim et al., 2003). Lipofectamine (Invitrogen, CA) was used as transfection agent, following the manufacturer's instructions. Renilla luciferase vector was used as an internal control for transfection efficiency. After transfection, cells were placed in complete medium for 24 h prior to TNF- α (20ng/ml) treatment for 6h. The cells were then washed twice with Phosphate-buffered Saline (PBS) and dissolved in 100 µl of lysis buffer. Cells were assayed for luciferase activity using the Dual-Luciferase assay system (Promega,

WI) and a TD 20/20 luminometer (Turner Designs, CA) according to the manufacturer's specifications. Individual assays were normalized for Renilla luciferase and data are expressed as the fold-increase in activity over unstimulated cells. Data are from three independent experiments, assayed in triplicate with comparable results. Chromatin immunoprecipitation (ChIP) assays were performed using Egr-1, SP1 and NF- κ B p65 antibodies from SantaCruz Biotechnology (SantaCruz, CA) and EZ-chip kit from Upstate (Temecula, CA), according to the manufacturer's directions. PCR analysis was performed using the following primers which span the NF- κ B and SP1/Egr1 sites at MIP-2 promoter- forward, 5' CAG GGC AGT AGA ATG AGG CAG G3', and reverse, 5'AGG CTG AAG TGT GGC TGG AGT C 3'. Control PCR analysis was also performed on input DNA for each sample.

3. Results

3.1. Characterization of gene expression profile in alveolar epithelial cells by CD8⁺ T cell recognition

CD8⁺ T cell mediated lung injury is initiated by antigen-specific recognition of epithelial antigen, resulting in expression of effector activities by the activated CD8⁺ T cells, after formation of the immunological synapse (Dustin, 2009). To better understand the gene expression changes that occur in alveolar epithelial cells triggered by CD8⁺ T cell recognition, we initially performed a microarray study utilizing an *in vitro* co-culture model consisting of MLE-Kd lung epithelial cells loaded with synthetic peptide (corresponding to the Kd-restricted 210–219 epitope of the A/Japan/57 HA) in the presence of HA-specific CD8⁺ T cells for 4 or 6 hr. MLE-Kd cells were then separated from T cells using CD8⁺ magnetic bead selection and RNA prepared from the epithelial cells were subjected to microarray analysis. Gene expression analysis revealed that about 70 and 100 genes were significantly induced, by more than 2-fold at 4 and 6 h, respectively, in MLE-Kd cells. We have listed the top 50 genes that are significantly up-regulated by CD8⁺ T cell recognition and showed distinct patterns of induction at 4 and 6 h (Table 1). The most highly induced genes in lung epithelial cells after CD8⁺ recognition were cytokine regulated, notably by TNF- α or IFN- γ . These results were consistent with the known effector functions of activated CD8⁺ T cells as well as our previous *ex-vivo* gene array results in mouse lung type II epithelial cells after CD8⁺ T cell transfer (Ramana et al., 2006). Interestingly, as a class, the chemokine genes were the most highly up-regulated. A second group of inducible genes include transcriptional factors. Genes predominantly induced by IFN- γ or TNF- α were listed in Table as group 3 and group 4, respectively. Cytokine signaling often involves induction of inhibitors that are involved in negative feed-back regulation. Thus, the induction of negative regulators of TNF- α signaling such as A20, ZFP-36 (tristetraprolin) was observed. We have also observed induction of genes involved in growth factor and G-Protein signaling (group 5 and group 6), the functional significance of which is not known at this time.

3.2. Activation of ERK1/2 and Egr-1 pathway by TNF- α and CD8⁺ T cell recognition in vitro and in vivo

TNF- α , a major cytokine produced by activated CD8⁺ T cells in both trans membrane and soluble form is known to activate MAP Kinases such as JNK, ERK, p38 as well as activation of transcription factors such as NF- κ B, AP1 and Egr-1 (Ghosh and Karin, 2003; Thiel and Cibelli, 2002). Time-course analysis by RT-PCR revealed that Egr-1 RNA levels was induced rapidly and transiently in MLE-Kd cells within 2–4 hours after CD8⁺ T cell recognition. TNF- α but not IFN- γ treatment, also rapidly induced Egr-1 expression (Figure 1A). Furthermore, adoptive transfer of HA-specific CD8 T cells into wild-type SPC-HA mice expressing HA antigen exclusively in alveolar epithelial cells also induced epithelial Egr-1 expression (Figure 1B). Activation of ERK by phosphorylation has been shown to induce Egr-1 in response to multiple stimuli in various cell types (Guha et al., 2001; Gineitis and Treisman, 2001). We tested whether CD8⁺ T cell recognition induced ERK activation in lung epithelial cells. ERK

activation as measured by western blot analysis with phospho-specific antibody was rapidly and transiently induced in MLE-Kd cells and in lung epithelial cells after CD8⁺ T cell recognition (Figure 2A and 2B). Importantly, ERK activation preceded Egr-1 induction. These results suggest that CD8⁺ T cell recognition stimulate ERK activity and Egr-1 induction *in vitro* and *in vivo*.

3.3. Inhibition of ERK activation abrogates Egr-1 induction and MIP-2 chemokine production by TNF- α or CD8⁺ T cell recognition

ERK 1/2 activation and Egr-1 induction have been shown to up-regulate the levels of cell adhesion molecules, cytokines and chemokines such as MCP-1 and MIP-2 in response to stress signals such as bacterial lipopolysaccharide, ischemia-reperfusion lung injury (Guha et al., 2001; Yan et al. 2000). We focused our attention on MIP-2 because this chemokine is involved in neutrophil influx into the alveolar space, an important component of CD8⁺ T cell mediated lung injury (Enelow et al., 1998). We tested whether ERK inhibitor U0126 suppressed Egr-1 induction and its downstream target MIP-2. MLE-Kd cells were pre-treated with 50 μ M U0126 for 2 hours and then stimulated with TNF- α for 0.5, 1 or 3.5 hours. RT-PCR analysis revealed that TNF- α induction of Egr-1 was significantly abrogated by pre-treatment with the ERK inhibitor, U0126 (Figure 3A). Dose response studies revealed that ERK inhibition by 25–50 μ M of U0126 resulted in suppression of Egr-1 and MIP-2 production in response to TNF- α or CD8⁺ T cell recognition (Figure 3B and 3C). Therefore it is likely that ERK activation is critical for the induction of Egr-1 and MIP-2 production. Furthermore, CD8⁺ T cells lacking TNF- α (TKO) failed to induce Egr-1 and MIP-2 in MLE-Kd cells (Supplementary data, Figure 1).

3.4. Egr-1 is required for MIP-2 production in response to TNF- α

To test the role of Egr-1 in MIP-2 transcriptional regulation, reporter constructs containing wild-type (WT) MIP-2 gene promoter, linked to luciferase, or the promoter with site specific mutations in the NF- κ B or Egr-1 consensus binding sites were transiently transfected into MLE-Kd cells (Figure 4A). TNF- α treatment stimulated luciferase activity of the WT MIP-2 promoter, whereas mutation of either NF- κ B or Egr-1 sites suppressed promoter activity by more than 3-fold (Figure 4B). In order to confirm the Egr-1-dependence on TNF- α -induced MIP-2 expression, we tested whether knock-down of Egr-1 by short-interfering RNA (si-RNA) has a significant impact on MIP-2 production. Western blot analysis revealed that Egr-1 si-RNA at a concentration of 25–50nM was effective in suppressing TNF- α mediated induction of Egr-1 protein, while control si-RNA has no effect (Supplemental Figure S2). ELISA analysis revealed that knock-down by Egr-1 specific si-RNA suppressed TNF- α mediated MIP-2 production in a dose-dependent manner (Figure 4C). Collectively, these results indicate that Egr-1 plays an important role in MIP-2 production.

3.5. Egr-1 binds to MIP-2 gene promoter *in vivo*

In order to determine whether Egr-1 plays a direct or indirect role in MIP-2 transcription, we assessed specific promoter binding triggered by TNF- α signaling. Previous studies have shown that Egr-1 directly regulates the gene promoter activity of chemokines MCP-1 (Ccl2) and IL-8, the human homolog of MIP-2 in response to interleukin 1 (Hoffman et al., 2008). Functional NF- κ B sites were also identified in these promoters (Kim et al., 2003; Wolter et al., 2008). We therefore performed chromatin immunoprecipitation on MLE-Kd lysates with Egr-1, NF- κ B (p65) and SP1 antibodies. As shown in Figure 5, TNF- α treatment induced Egr-1 and p65 binding to the MIP-2 gene promoter, though p65 binding was more rapid and preceded Egr-1 binding to MIP-2 gene promoter. SP1 was constitutively bound to MIP-2 gene promoter in untreated cells and this recruitment was not significantly altered by TNF- α treatment.

3.6. Role of Egr-1 in CD8⁺ T- cell mediated lung injury

We have shown that the initiating event in lung injury is antigen-specific expression of TNF- α by CD8⁺ T cells upon recognition of alveolar epithelial viral antigen (HA) and the expression of chemokines mediated by TNFR receptor subunits R1 and R2 on epithelial cells (Xu et al., 2004; Liu et al. 2005). Activation of transcription factors such as NF- κ B, AP1 and Stat1 in epithelial cells may play an important role in chemokine gene expression and the resultant inflammatory influx which contributes significantly to the severe lung injury (Ramana et al., 2006). To directly test the role of Egr-1 in CD8⁺ T- cell pulmonary immunopathology in a model of respiratory infection (without the complicating factors of a replicating virus), we have generated wild-type and Egr-1 KO mice expressing HA antigen under the control of the Surfactant Protein-C (SP-C) gene promoter resulting in alveolar epithelial expression. Adoptive transfer of 5X10⁶ HA-specific CD8⁺ T cells into wild-type HA+ mice resulted in severe lung injury characterized primarily by infiltration of neutrophils and macrophages. In contrast, neutrophil as well as macrophage influx was significantly reduced and lung injury was dramatically abrogated in Egr-1 KO HA+ mice after CD8⁺ T cell transfer (Figure 6). The histopathology of lungs from wild-type HA+ mice showed dense interstitial and intraalveolar mononuclear and polymorphonuclear infiltration, with hemorrhage and edema (Figure 6A). The inset is the high-power view of the histology in Figure 6A. The Egr-1 KO HA+ mice, in contrast, had minimal inflammatory infiltration throughout the lung fields, and the occasional small areas of inflammation indicated by arrow in Figure 6B were remarkable for the absence of polymorphonuclear neutrophils (the cells shown in the inset are largely eosinophils, which were not evident at all in the wild-type lungs).

We have used a chemokine gene array containing more than 100 genes to characterize differences in gene expression between wild-type and Egr-1 KO lung epithelial cells, harvested 6 hr after CD8⁺ T cell transfer (Figure 7A). This analysis revealed that a large number of chemokine and inflammatory genes including Ccl2 (MCP-1), Ccl4 (MIP-1), Ccl6, Ccl9, Ccl17 (TARC), CCR1, CCR2, Cxcl2 (MIP-2), Cxcl4 (PF-4), Cxcl7 (NAP-2) and TLR-4 were induced in WT-HA+ and suppressed in Egr-1 KO HA+ mice. We have confirmed the array results with RT-PCR for MIP-2 and MCP-1 (Figure 7B).

4. Discussion

Infection with highly pathogenic strains of influenza result in considerable immunopathologic lung injury, a large component of which may be T cell -mediated (Bruder et al., 2006). T-lymphocytes also infiltrate the lung in a variety of human disease states, such as sarcoidosis, hypersensitivity pneumonitis and other infections characterized by acute and chronic lung injury (Leatherman et al., 1984; Harty et al., 2006). The interaction between the CD8⁺ TCR and peptide/MHC complex on alveolar epithelial cells is an important determinant of viral clearance as well as the inflammatory response. We have shown that CD8⁺ T cell recognition of alveolar antigen triggered expression profiles of TNF- α -dependent and IFN- γ -dependent genes in epithelial target cells. Lung injury was dependent upon TNF- α ; expressed by the CD8⁺ T cell, and by chemokines expressed by the antigen-presenting epithelial cells upon T cell recognition (Xu et al., 2004). In this study, we have used a simple *in vitro* co-culture system of MLE-Kd lung epithelial cells to examine the gene expression profile induced in MLE-Kd cells by CD8⁺ T cell recognition and found it was dominated by TNF- α ; and IFN- γ regulated genes, as we had previously observed *in vivo* (Ramana et al., 2006). These include chemokines such as MIP-2, KC, MIP-1 β , MCP-1, MIG and IP-10, which are potent chemoattractants of neutrophil, macrophage and T cells that are involved in CD8⁺ T cell mediated lung injury (Zhao et al., 2000). Induction of transcription factors such as IRF-1, AP1 (Fos, Jun), NF- κ B and Egr-1 may potentiate cytokine responses (Ramana et al., 2000; Baud and Karin., 2001; Cao et al., 1992). Furthermore, negative regulators such as A20 and ZFP-36 may contribute to

the termination of the epithelial signal transduction after CD8 T cell recognition (Beyaert et al., 2000; Sandler and Stoecklin, 2008). This co-culture system provides an important model to dissect early events in signal transduction in CD8⁺ T cell recognition which are very difficult to accomplish *in vivo*. However, the number of genes induced in MLE-Kd cells was significantly higher and much more diverse (greater than 100 genes in MLE-Kd vs 35 in mouse lung type II cells *ex vivo* at 6h) in response to CD8⁺ T cell recognition. This difference may be due to several factors including effector-target (E:T) ratios achieved in cell culture compared with the *in vivo* circumstances, the requirement of transit time for CD8⁺ T cells to accumulate in the lungs for CD8⁺ T cell recognition and the asynchronous nature of *in vivo* events (Galkina et al., 2005).

Recent studies have revealed a set of primary response genes (PRG) for TNF- α and Toll-like receptors (TLR) signaling that are rapidly inducible by transcription and independent of new protein synthesis (Ramirez-Carrozzi et al., 2009; Hargreaves et al., 2009). A major subset of the primary response genes (group A) including Egr-1 and MIP-2 contain CpG islands in the gene promoters and features of actively transcribed genes such as histone modifications, SP1 binding and recruitment of RNA polymerase II and TATA binding protein (TBP) in unstimulated cells. These genes are induced by TNF- α and TLR signaling independent of nucleosome remodeling. Analysis of our microarray data revealed that about 16 genes were TNF- α mediated primary response genes and among them, 9 belonged to group A.

Activated CD8⁺ T cells secrete a variety of cytokines including IFN- γ and TNF- α upon antigen recognition leading to the target epithelial cell activation of NF- κ B, AP1 and Stat1 transcription factors in lung epithelial cells (Ramana et al., 2006). We have shown that Egr-1 is rapidly and transiently induced in MLE-Kd cells by CD8⁺ T cell recognition or TNF- α and that adoptive transfer of HA-specific CD8⁺ T cells into SPC-HA+ mice also induced Egr-1 in lung epithelial cells. In both cases, ERK activation by CD8⁺ T cell recognition preceded Egr-1 induction. The core promoter region of Egr-1 contains multiple serum response elements (SRE) that are bound constitutively by serum response factor (SRF) and one member of the ternary complex factor (TCF) family that includes Elk-1, SAP1 and NET (Chai and Tarwinski, 2002). Activated ERK undergoes rapid nuclear translocation resulting in Elk-1 phosphorylation and transcriptional activation of Egr-1 (Guha et al., 2001). Previous studies have shown that a wide spectrum of agents including serum, growth factors, cytokines as well as cytotoxic chemicals such as lipopolysaccharide (LPS), arsenic and carbon monoxide stimulate ERK activation and Egr-1 induction (Kaufmann and Theil 2001; Gineitis and treisman, 2001; Al-Sarraj and Thiel, 2004; Mishra et al., 2006). Consistent with these observations, inhibition of ERK activation by Mek-1 inhibitor U0126 severely impaired Egr-1 induction by TNF- α . We have shown that NF- κ B and Egr-1 bind to specific elements in MIP-2 gene promoter and mediate the transactivation. Functional interactions between SP1 and Egr-1 zinc finger transcription factors on natural and synthetic overlapping G+C-rich elements sites and their role in transcriptional regulation have been demonstrated (Huang et al., 1997; Zhang et al., 2007). Our results indicate that unlike NF- κ B and Egr-1, SP1 binds constitutively to MIP-2 gene promoter in untreated and TNF- α treated cells. However, these results do not preclude a regulatory role for SP1, especially in untreated cells. Current models of inducible transcription by TNF- α and TLR signaling include an important role for SP1 in preventing inappropriate induction of primary response genes containing CpG islands. It has been shown that SP1-recruited RNA polymerase II generates unspliced transcripts that are rapidly degraded in untreated cells whereas NF- κ B p65 recruitment facilitates additional chromatin modifications that couple elongation of polymerase II and RNA processing machinery resulting in productive transcription (Ramirez-Carrozzi et al., 2009; Hargreaves et al., 2009; Singh, 2009).

CD8⁺ T cells lacking TNF- α (TKO) failed to induce Egr-1 and MIP-2 in MLE-Kd cells. Previous studies have shown that compared with wild-type (WT), TKO CD8⁺ T cells were

dramatically less injurious to the mouse lung (Xu et al., 2004). Taken together, these results suggest a critical role of TNF- α in alveolar epithelial Egr-1 induction and in CD8⁺ T cell mediated lung injury. Si-RNA knockdown of Egr-1 severely impaired MIP-2 production in MLE-Kd cells, in response to TNF- α . Further studies are required to demonstrate functional interactions between SP1 and Egr-1 in the regulation of MIP-2 in response to TNF- α .

Egr-1 regulates a large number of genes such as cell adhesion molecules, cytokines and chemokines involved in inflammatory response and lung injury (Yan et al., 2000; Pawlinski et al., 2003). Chemotactic cytokines or chemokines are a large superfamily of structurally and functionally related molecules that induce directional migration and activation of specific leukocyte population from the vasculature into the tissue during inflammation (Charo and Ransohoff, 2006). There are at least 50 distinct chemokines which are 70–90 amino acids in length, and are divided into four subfamilies, C, CC, CSC and CXC, based on their conserved cysteine residues located near the amino terminus (Viola and Luster, 2008). Chemokine array analysis revealed that Egr-1 plays a major role in inducing chemokines and their receptors such as Ccl2 (MCP-1), Ccl17(TARC), Endothelial monocyte-activating polypeptide (EMAP-2), CCR1 and CCR5 that are involved in mononuclear influx as well as Cxcl2 (MIP-2), Ccl6 and Ccl9 that are involved in neutrophil migration to the lung (38). Ccl2 and CCR2 are involved in inflammatory diseases of the lung such as allergic asthma, pulmonary fibrosis and bronchiolitis (Roe et al., 2003). EMAP-2 is a pro-inflammatory cytokine induced in LPS mediated lung inflammation (Journeay et al., 2007). MIP-2 is involved in neutrophil recruitment and is elevated in a variety of models of lung injury (Yan et al., 2000; Shanley et al., 1997). CXCR2, the chemokine receptor for MIP-2 has been shown to be required for neutrophil trafficking to the lung during influenza virus infection (Wareing et al., 2007). Ccl6 and Ccl9 are chemoattractants for CD11b⁺ dendritic cells (Zhao et al., 2003; Coelho et al., 2007). The CD18 integrin Mac1 (CD18/CD11b⁺) is required for neutrophil recruitment into the lung (Moreland et al., 2002). In Egr-1 KO-HA⁺ mice, expression levels of these chemokines and signaling molecules were impaired after CD8⁺ T cell recognition and host neutrophil and mononuclear influx were decreased resulting in milder lung injury.

Egr-1 has been shown to play an important role in acute lung injury (Ngiam et al., 2007). Significantly, Egr-1 gene deletion diminished vascular injury in murine models of ischemia and reperfusion by decreased cytokine and chemokine production and enhanced animal survival (Yan et al., 2000; Prince et al., 2007). Our results demonstrate a similarly important role in CD8⁺ T cell mediated lung injury, which may underly the immunopathology observed in the context of severe influenza infection, as well as in other viral and inflammatory lung diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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(A)

(B)

Figure 1. Induction of Egr-1 by CD8⁺ T cell recognition or TNF-α treatment in MLE-Kd cells
 (A) MLE-Kd cells were co-cultured with CD8⁺ T cells at an effector: target (E/T) ratio of 2:1 in the presence of HA peptide for 2,4 or 6h or treated with TNF-α (20 ng/ml) or IFN-γ (100U/ml) for 1h. MLE-Kd cells were separated from CD8⁺ T cells by magnetic selection and RNA levels of Egr-1 and β-actin were determined by RT-PCR analysis. (B) RNA was prepared from lung type II cells at 0, 2, 4, 6h after adoptive transfer of HA-specific CD8⁺ T cells into SPC-HA mice. Egr-1 and β-Actin levels were determined by RT-PCR analysis. The results shown are representative of three independent experiments.

(A)

(B)

Figure 2. Activation of extracellular signal-regulated kinases (ERK) by CD8⁺ T cells in MLE-Kd cells *in vitro* and epithelial cells *in vivo*

(A) MLE-Kd cells were co-cultured with CD8⁺ T cells and HA peptide for 0, 0.5, 1, 2h. MLE-Kd cells were separated from CD8⁺ T cells. Cell extracts were prepared from MLE-Kd and the protein levels of phosphorylated (p-ERK) and total ERK levels were determined by western blot analysis. (B) Cell extracts were prepared from lung type II cells at 1, 2, 4h after adoptive transfer of HA-specific CD8⁺ T cells into SPC-HA⁺ mice. Protein levels of phosphorylated (p-ERK) and total ERK levels were determined by western blot analysis. The results shown are representative of three independent experiments.

Figure 3. Inhibition of ERK activation abrogates TNF- α mediated Egr-1 and MIP-2 induction in MLE-Kd cells

(A) MLE-Kd cells were left untreated or pre-treated with 50 μ M of Mek-1 inhibitor U0126 for 2h and stimulated with TNF- α for an additional 0.5, 1, 3.5h. Total RNA was isolated and Egr-1 and β -Actin mRNA expression levels were determined by RT-PCR analysis. The results shown are representative of three independent experiments.

(B) Cells were pre-treated with 25 or 50 μ M of U0126 for 2h and then stimulated with TNF- α for an additional 1h. Total RNA was isolated and Egr-1, MIP-2 and β -Actin mRNA levels were determined by RT-PCR analysis. The results shown are representative of three independent experiments.

(C) MLE-Kd cells were left untreated or pre-treated with indicated concentration of U0126 for 2 hr. The cells were washed with serum-free medium and treated with TNF- α for an additional 4h. Culture supernatants were collected and assayed for MIP-2 production using ELISA. Data are presented as the means $\pm$ S.E.M. of three independent experiments. Significance was determined using ANOVA ($p < 0.05$ was considered significant).

Figure 4

Figure 4.

Role of Egr-1 in the regulation of MIP-2 expression by TNF- α in MLE-Kd cells. (A) A Schematic representation of the pMIP-2(-187)-luciferase wild-type plasmid and constructs with mutation in the NF- κ B or Egr-1 elements. (B) Plasmid constructs were transfected into MLE-Kd cells for 36h and cells were left untreated or treated with TNF- α (20ng/ml) for 6h. Luciferase activity measurements of each construct were done as described in materials and methods. Data are shown as mean $\pm$ S.D. (n=3; $p < 0.01$).

(C) MLE-Kd cells were transfected with different amounts of Egr-1 Si-RNA for 36h and cells were left untreated or treated with TNF- α (20ng/ml) for 6h. MIP-2 levels in the culture medium were determined by ELISA. Data are presented as the means $\pm$ S.E.M. of three independent experiments ($p < 0.05$).

Figure 5. Egr-1 and NF-kB (p65) interacts with MIP-2 gene promoter *in vivo*

Chromatin was immunoprecipitated with Egr-1 NF-kB (p65) or SP1 antibodies from MLE-Kd cells left untreated or treated with TNF- α for 15 or 30 min. Recovered genomic DNA was amplified with primers specific for MIP-2 promoter region. As a control, genomic DNA was extracted from the samples (input) and amplified with primers specific for MIP-2 promoter region. The results shown are representative of 2 independent experiments.

Figure 6. Lung immunopathology in WT and Egr-1 KO HA+ mice after adoptive transfer of HA-specific CD8⁺ T cells

HA-specific 5×10^6 CD8⁺ T cells were transferred into WT and Egr-1 KO HA+ mice by tail vein injection. Lung sections from mice were stained with hematoxylin and eosin (H&E), 5 days after CD8⁺ T cell transfer. Original magnification was X10 and the inset X40. The arrow in (B) refers to a small focus of peribronchial eosinophil influx in Egr-1 KO HA+ mice. These figures are representative of two independent experiments, with 3 WT and 3 Egr-1 KO HA+ mice each.

Figure 7. Chemokine expression in WT and Egr-1 KO HA+ mice after adoptive transfer of HA-specific CD8⁺ T cells
 (A) Chemokine gene expression in lung epithelial cells of WT and Egr-1 KO HA+ mice was analyzed 6h after CD8⁺ T cell transfer, using chemokine arrays from Superarray. Relative RNA levels in WT and Egr-1 were quantitated and expressed as fold suppression in Egr-1 KO compared to WT alveolar epithelial cells (B) Confirmation of array results of MIP-2 and MCP-1 by RT-PCR.

Table 1

Gene induction in MLE-Kd cells in vitro after CD8+ T cell recognition

Functional Category	Gene name	Fold 4 hr	change 6 hr
1. Chemokine	Monokine induced by gamma interferon (MIG/Cxcl9))	708	420
	Monocyte chemoattractant protein (MCP-1/Ccl2)	201	103
	Macrophage inhibitory protein (MIP1-beta/Ccl4)	100	126
	Interferon inducible protein (IP-10/Cxcl10)	51.8	15.5
	Melanoma growth factor (KC/Cxcl1)	34.1	13.4
	Macrophage inhibitory protein (MIP-2/Cxcl2)	8.2	8.6
	Small inducible cytokine D1 (Fractalkine/Cx3cl1)	3.1	1
2. Transcription	Interferon regulatory factor (IRF-1)	13.9	4.3
	Interferon inducible protein LRG21 (ATF3)	12.4	7.7
	Fos related antigen (Fra1)	3.9	3.1
	c-Jun	6.4	4.5
	Jun-B	1	4.7
	Rel p65	5.5	8.8
	NF-kB p105	3.6	8.7
	c-Myc	3	3.8
	C/EBP delta	2.4	1
	Early growth response-1 (Egr-1)	4.5	2
3. IFN signaling	IFN inducible Gaunylate binding protein (GBP-2)	3.1	2.5
	IFN inducible GTPase (Mg11)	2.4	1.7
	IFN inducible p204	6.5	3.3
	IFN induced tetratricopeptide (IFIT-1)	2.6	1
	IFN inducible p202	3	1
	IFN induced tetratricopeptide (IFIT-2)	5.6	2.8
	IFN inducible GTPase (IIGP)	2	1.3
	Cytokine inducible SH2 domain (CISH)	3	4.5
	Indoleamine 2,3-dioxygenase (IDO)	2.7	5.1
4. TNF signaling	Nitric oxide synthase 2 (NOS-2)	11.9	9.7
	Cyclooxygenase 2 (COX-2/Ptgs2)	5.1	3.9
	Serum amyloid A3(SAA-3)	8.8	1
	Prostaglandin E receptor (EP4 subtype)	3.2	6.9
	Apoptosis inhibitor 2 (cIAP2)	1	3.6
	Glycoprotein 49A	6	10.6
	Glycoprotein 49B	5	18
	Serine protease inhibitor 6	3	25.6
	Tissue inhibitor of metalloproteinase (TIMP-3)	15.2	11.7
	A disintegrin and metalloproteinase (ADAM-8)	13.2	1
	TNF-induced protein 3 (A20/Tnfaip3)	18	1
	ZFP-36 (Tristetraprolin)	4.7	1
	I-kB beta	5.6	4.8
	I-kB epsilon (Ikbke)	5.6	10.2
	Intercellular adhesion molecule (ICAM-1)	2.1	2.2
5. Growth factor signaling	Fibroblast inducible secreted protein (FISP)	5.5	3.4
	Epiregulin	7.2	1
	Insulin growth factor binding protein (IGFBP10)	10.5	1
	Immediate early (gly96)	4.2	1.9
	RhoB	8	2.4
	Tyrosine-threonine phosphatase (PAC-1)	3.2	7.9
	Thrombospondin1	11.5	9.1
6. G-protein	G-protein coupled receptor (TDAG8; GPR65)	12.3	55