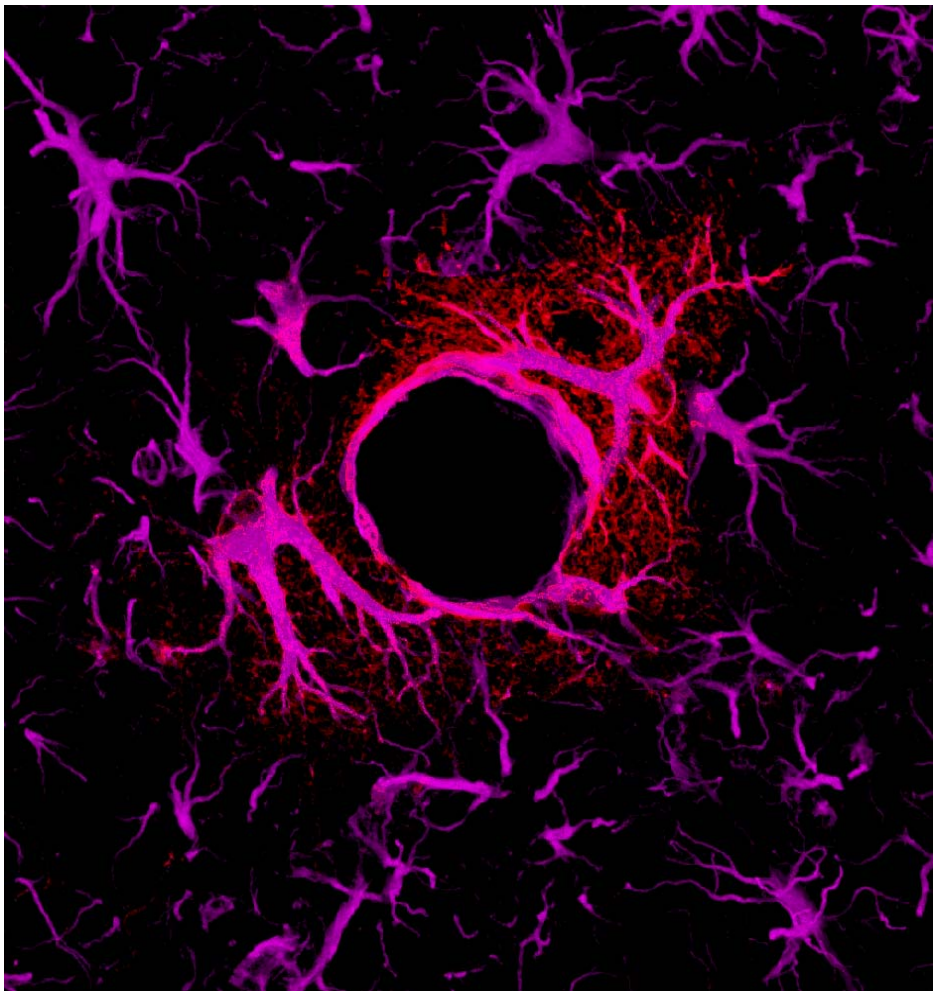

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Rapid Upregulation of Interferon-Regulated and Chemokine mRNAs upon Injection of 10^8 International Units, but Not Lower Doses, of Adenoviral Vectors into the Brain

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The innate immune response, characterized by the rapid induction of proinflammatory genes, plays an important role in immune responses to viral vectors utilized in gene therapy. We demonstrate that several innate proinflammatory mRNAs, i.e., those coding for the interferon (IFN)-regulated proteins interferon regulatory factor 1, 2′,5′-oligoadenylate synthetase, and double-stranded-RNA-dependent protein kinase as well as those coding for the chemokines RANTES, IFN-γ-inducible protein 10, and monocyte chemoattractant protein 1, were all increased in a statistically significant manner in response to 1×10^8 IU, but not lower doses, of a first-generation adenovirus injected into the naïve brain. This indicates the presence of a threshold dosage of adenovirus needed to elicit an acute innate inflammatory response.

The innate immune response is the first line of defense against viral infection and is also stimulated upon infection with virus-derived gene transfer vectors. Interferons (IFNs) and IFN-regulated genes produced by infected cells induce enzymes capable of degrading incoming viral mRNAs, blocking viral protein synthesis, and eventually killing infected cells (7). In addition, chemokines provide signals for the recruitment of numerous cell types into infected tissues (5, 15–17, 21, 22, 27). Adenoviral vector (Adv) infection of the liver is well characterized and results in an influx of neutrophils, monocytes, macrophages, and NK cells and the induction of inflammatory genes, i.e., the genes for tumor necrosis factor alpha, IFN-γ, interleukin-6 (IL-6), IL-12, and IL-1β (11, 12). Similarly, injection of Adv into the brain causes a dose-dependent infiltration of macrophages, neutrophils, lymphocytes, and NK cells (2–4, 23–25). However, the dose response and time course and the roles of interferons and chemokines in the innate immune response to the injection of adenovirus into the brain remain unknown. In the liver, innate immune responses reduce viral input genomes >90% in under 24 h (30). Thus, the effects of adenoviral vectors on innate immune responses in the central nervous system (CNS) deserve further consideration.

We utilized RAd35 (a human cytomegalovirus carrying *lacZ*), a first-generation adenoviral vector that is described elsewhere (8, 18, 19, 29). C57BL/6 mice were anesthetized using ketamine (75 mg/kg of body weight) and medetomidine (0.5 mg/kg) and injected in the right striatum with 1×10^5 to 1×10^8 infectious units (particle/infectious-unit ratio, 30) of RAd35 (free of endotoxin and replication-competent adenovirus) or saline in 0.5 μl, sacrificed, and perfused with oxygenated Tyrode's solution alone or followed by 4% paraformaldehyde in saline solution for immunohistochemistry. RNAs were isolated from the striatal injection site by using TRIzol (Invitrogen Technologies). Probe sets for chemokines and IFN-regulated genes and RNase protection assays (RPAs) have been described previously (1). Autoradiographs were scanned and band densities assessed using ImageJ software (NIH, Bethesda, MD). The value for each band was compared to the L32 control band value in the corresponding lane and expressed as a ratio. Films were exposed overnight to obtain a correct reading exposure of the L32 bands, in contrast to the longer exposures needed for the correct detection of mRNAs of interest. Statistical analysis was done using two-way analysis of variance followed by a Tukey-Kramer multiple comparison test (NCSS software).

In saline-injected mice, only the IFN-regulated genes interferon regulatory factor 2 (IRF-2) and p58 were expressed at basal levels at 1, 3, and 7 days postinjection. Injection of 1×10^5 IU did not increase the expression of any IFN-regulated genes over basal levels, and following injection of 1×10^6 IU, only IRF-1 showed a small increase at 1 day postinjection. After injection of 1×10^7 IU, there were small increases in IRF-1, 2′,5′-oligoadenylate synthetase (OAS), T-cell-specific guanine nucleotide triphosphate-binding protein (TGTP), and double-stranded RNA (dsRNA)-dependent protein kinase (PKR) at 3 and 7 days; however, none of these were significantly higher than those of controls. Following injection of 1×10^8 IU, however, we detected a statistically significant increase in expression of mRNAs coding for IRF-1, OAS, and PKR. The levels of these mRNAs remained elevated for up to 7 days (Fig. 1a to d).

Our results demonstrate that whereas IRF-2 and p58 appeared to be constitutively expressed and not regulated by adenovirus injection, the IFN-regulated mRNAs for IRF-1, OAS, and PKR were found to have significant increases in expression and therefore may play a critical role in the innate immune response to the

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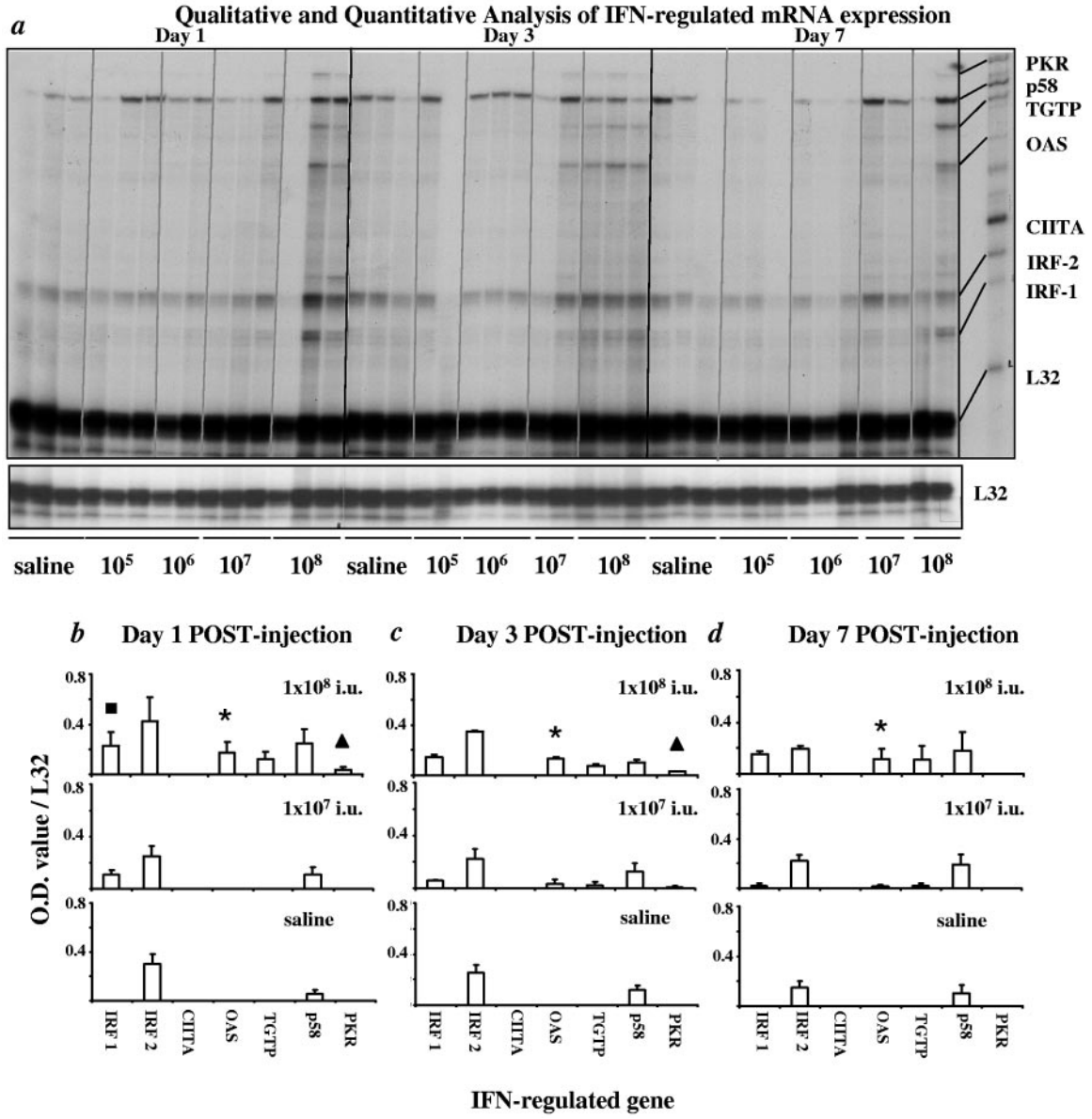

FIG. 1. Analysis of IFN-regulated mRNA expression. (a) RPA gel showing bands corresponding to mRNAs of IFN-regulated mRNAs 1 day (left panel), 3 days (middle panel), and 7 days (right panel) after intracranial injection of RAd35. Dosages of RAd35 (1×10^5 to 1×10^8) or saline are shown below the lanes. Sizes of individual IFN-regulated mRNA probes are indicated at the far right. (b to d) Quantification of IFN-regulated mRNA expression using ImageJ software. The band intensity was determined by dividing the optical density (OD) value for each IFN-regulated mRNA by the OD value for the L32 control in each lane and was expressed as a percentage. To obtain a representative reading of the denser L32 bands, these bands were exposed overnight (lower L32 image), while for readings from the other mRNAs, the gels were exposed for 5 days. (b) Quantification of IFN-regulated mRNA expression 1 day after CNS injection of saline or 1×10^7 to 1×10^8 IU of RAd35. (c) Quantification of IFN-regulated mRNA expression 3 days after CNS injection of saline or 1×10^7 to 1×10^8 IU of RAd35. (d) Quantification of IFN-regulated mRNA expression 7 days after CNS injection of saline or 1×10^7 to 1×10^8 IU of RAd35. *, $P < 0.1$ compared to control (saline) OAS value; ▲, $P < 0.1$ compared to control PKR value; ■, $P < 0.1$ compared to control IRF-1 value at each time point. Quantification of expression after the injection of 1×10^5 to 1×10^6 IU of virus is not illustrated because changes were not statistically significant. We only show expression below (1×10^7 IU) and above (1×10^8 IU) the threshold of induction of statistically significant increases in mRNA expression.

highest dose, 1×10^8 IU, of RAd35. The peak of expression of IFN-regulated genes occurred at 24 h postinjection, but expression continued to be detected for up to 7 days, indicating an early role for interferons and IFN-regulated genes in the innate immune response to viral vectors. Thus, a threshold for the induction of an IFN-regulated innate immune response exists at 1×10^8 IU of adenovirus, since lower doses did not stimulate a statistically significant IFN response.

Because chemokines orchestrate inflammatory cell recruitment in both the liver (33) and CNS (14), we measured acute chemokine mRNA responses to RAd35 injection. In saline- and virus (1×10^5 IU)-injected mice, there was no expression

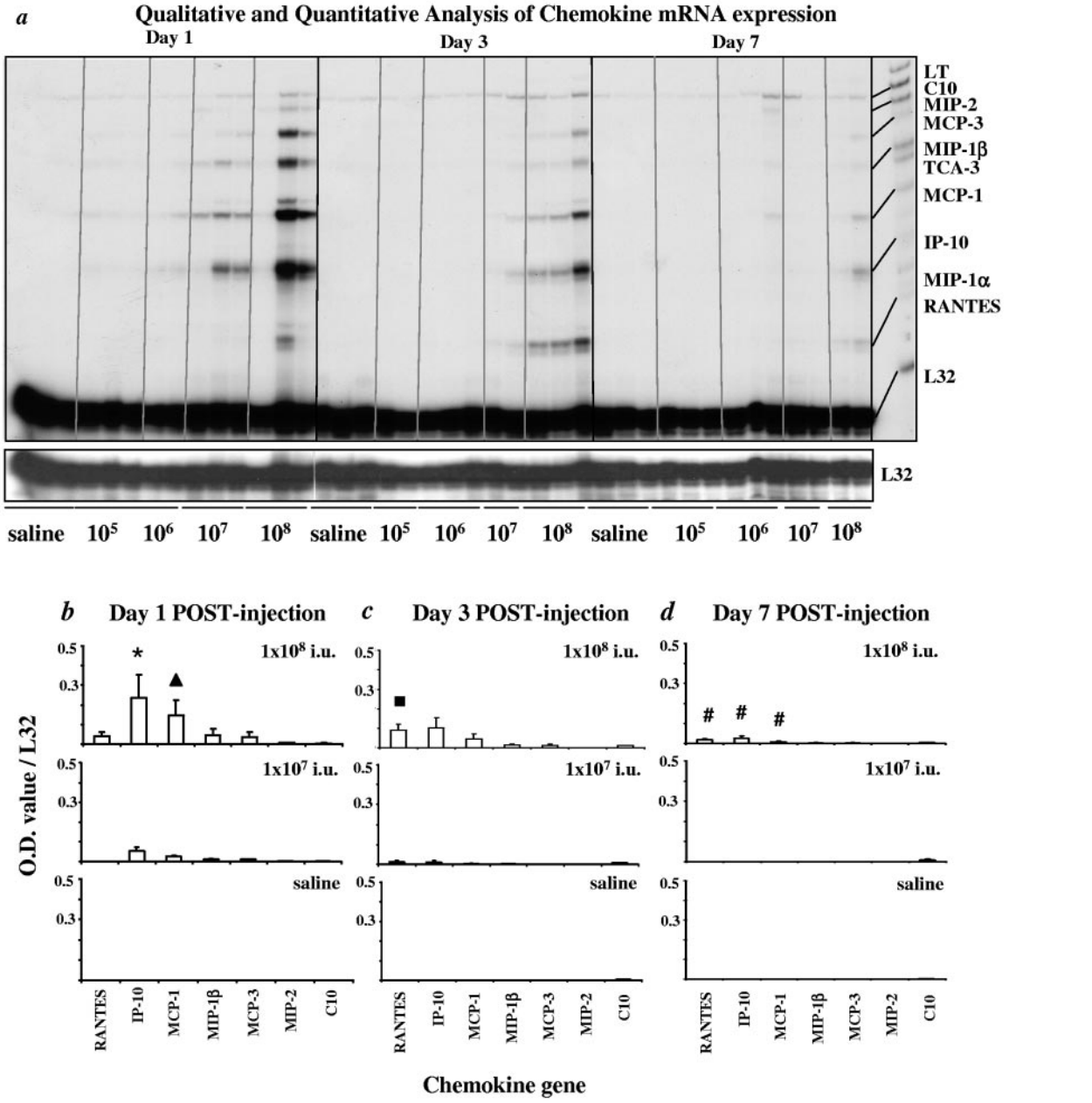

FIG. 2. Analysis of chemokine mRNA expression. (a) RPA gel showing bands corresponding to mRNAs of chemokine genes 1 day (left panel), 3 days (middle panel), and 7 days (right panel) after intracranial injection of RAd35. Dosages of RAd35 (1×10^5 to 1×10^8) or saline are shown below the lanes. Sizes of individual chemokine mRNA probes are indicated at the far right. (b to d) Quantification of chemokine mRNA expression using ImageJ software. The band intensity was determined by dividing the OD value for each chemokine mRNA by the OD value for the L32 control in each lane and was expressed as a percentage. To obtain a representative reading of the denser L32 bands, these bands were exposed overnight (lower L32 image), while for readings from the other mRNAs, the gels were exposed for 5 days. (b) Quantification of chemokine mRNA expression 1 day after CNS injection of saline or 1×10^7 to 1×10^8 IU of RAd35. (c) Quantification of chemokine mRNA expression 3 days after CNS injection of saline or 1×10^7 to 1×10^8 IU of RAd35. (d) Quantification of chemokine mRNA expression 7 days after CNS injection of saline or 1×10^7 to 1×10^8 IU of RAd35. ⋆, $P < 0.1$ compared to control (saline) IP-10 value; ▲, $P < 0.1$ compared to control MCP-1 value; ■, $P < 0.1$ compared to control RANTES value at each time point; #, $P < 0.1$ compared to day 1 RANTES, IP-10, and MCP-1 values after injection of 1×10^8 IU. Quantification of the injection of 1×10^5 to 1×10^6 IU of virus is not illustrated because changes were not statistically significant. We only show expression below (1×10^7 IU) and above (1×10^8 IU) the threshold of induction of significant increases in mRNA expression.

of chemokine mRNAs at any of the time points examined. Following intracerebral injection of 1×10^6 IU of virus, only low levels of the mRNAs for IFN-γ-inducible protein 10 (IP-10; also called CXCL10), monocyte chemoattractant protein 1 (MCP-1; also called CCL2), macrophage inflammatory protein 1β (MIP-1β; also called CCL4), monocyte chemoattractant protein 3 (MCP-3; also called CCL7), and MIP-related protein 1 (C10; also called CCL6) were expressed at 1 day postinjec-

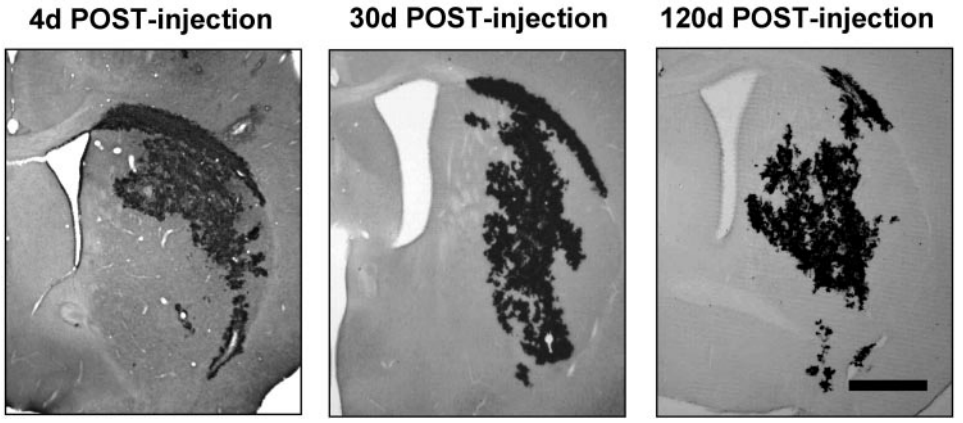

FIG. 3. Qualitative analysis of β-galactosidase (β-Gal) transgene expression. Qualitative analysis of the distribution of β-Gal-expressing cells in the CNSs of C57BL/6 mice 4, 30, and 120 days following injection of 1×10^7 IU of RAd β-Gal (an adenovirus carrying the β-Gal transgene) showed robust transgene expression over time. Scale bar, 1 mm.

tion, and only residual levels of C10 were detectable at 3 and 7 days postinjection. Injection of 1×10^7 IU resulted in higher expression of the chemokines IP-10, MCP-1, MIP-1β, MCP-3, MIP-2, and C10 at 1 day postinjection. RANTES (regulated on activation of normal T cell expressed and secreted; also called CCL5), IP-10, and C10 had small increases at 3 days postinjection, and only very low levels of C10 remained at 7 days postinjection. However, none of these changes achieved statistical significance compared to controls. Only at the highest dose, 1×10^8 IU, did we detect significantly elevated levels of IP-10, MCP-1, and RANTES (Fig. 2a to d). mRNAs for MIP-1β and MCP-3 were also elevated but did not reach statistical significance. Elevated mRNAs were significantly reduced at the 7-day time point. Thus, while the IFN-regulated response remained active for 7 days, the chemokine increase was transient.

The IFN-regulated genes that had the largest increases, i.e., those encoding OAS, IRF-1, and PKR, have several different functions. OAS activates the endoribonuclease RNase L to degrade single-stranded viral RNA (13), IRF-1 activates interferon alpha and beta transcription (20), and PKR is involved in phosphorylation and activation of the NF-κB pathway (10). OAS, IRF-1, TGTP, and PKR have each been implicated in the innate immune responses to viral infections in the liver (6, 28, 31, 32). Despite the rapid dose-dependent increase in expression of the IFN-regulated genes at early time points and their continued expression for up to 7 days following infection, transgene expression from adenoviral vectors remains unaffected, as expression can be detected by immunohistochemistry for up to at least 4 months after injection of adenoviral vectors into the brain (Fig. 3) (3, 8, 9, 23, 25, 26, 34).

The chemokines RANTES, MIP-1β, MCP-1, and MCP-3 have been shown to recruit monocytes, activated T cells, NK cells, and dendritic cells into infected tissues, while IP-10 is primarily involved in the recruitment of activated T cells and NK cells (5, 15–17, 21, 22, 27). Previous work from our laboratory has shown that following injection of 1×10^8 IU of adenoviral vector into the brain, infiltration of $CD45^+$ lymphocytes, monocytes, granulocytes, dendritic cells, and NK cells and of $F4/80^+$ macrophages and activated microglial cells can be seen at 7 days postinjection. The influxes of $CD45^+$ and $F4/80^+$ cells are consistent with the chemokine mRNAs that were elevated in our RPA studies and provide evidence that these may be involved in the recruitment of different immune cells into the CNS. Furthermore, the chemokine mRNAs with increased expression in the CNS following injection of RAd35 are similar to those seen to increase in the liver (MIP-1β, MIP-3, MCP-1, and IP-10) (33) and the spinal cord (RANTES, MCP-1) (14), indicating that similar sets of chemokines may be involved in recruitment of immune cells to the brain and to peripheral organs.

Our work demonstrates that specific chemokines and IFN-regulated genes are involved in the rapid inflammatory response to first-generation adenoviral vectors in the CNS, and it is this innate response that produces transient inflammation in the brain that could exacerbate preexisting conditions and eliminate vectors carrying beneficial transgenes. However, this increase was only statistically significant at a dose of 1×10^8 IU. Previously, we demonstrated that doses below, but not above, this threshold allow long-term transgene expression in the brain and induce relatively mild early innate inflammatory responses (23). We demonstrate here that the mechanism underlying the threshold is the stimulation of the IFN-regulated inflammatory response and gene expression and the activation of chemokine production. The elucidation of the threshold at which viral vectors induce a strong innate inflammatory response explains why the cellular inflammatory response is stronger and deleterious at doses of vector of $>1 \times 10^8$ IU and why these high doses will curtail long-term gene expression in the CNS. These findings are of importance for the future of adenoviral vector-mediated gene therapy, as they establish the dose of adenoviral vector below which a response from the innate immune system will not be initiated and the vector will thus be safe and effective (23).

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