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Immune Responses to Adenovirus and Adeno-Associated Vectors Used for Gene Therapy of Brain Diseases: The Role of Immunological Synapses in Understanding the Cell Biology of Neuroimmune Interactions

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Abstract: Researchers have conducted numerous pre-clinical and clinical gene transfer studies using recombinant viral vectors derived from a wide range of pathogenic viruses such as adenovirus, adeno-associated virus, and lentivirus. As viral vectors are derived from pathogenic viruses, they have an inherent ability to induce a vector specific immune response when used *in vivo*. The role of the immune response against the viral vector has been implicated in the inconsistent and unpredictable translation of pre-clinical success into therapeutic efficacy in human clinical trials using gene therapy to treat neurological disorders. Herein we thoroughly examine the effects of the innate and adaptive immune responses on therapeutic gene expression mediated by adenoviral, AAV, and lentiviral vectors systems in both pre-clinical and clinical experiments. Furthermore, the immune responses against gene therapy vectors and the resulting loss of therapeutic gene expression are examined in the context of the architecture and neuroanatomy of the brain immune system. The chapter closes with a discussion of the relationship between the elimination of transgene expression and the *in vivo* immunological synapses between immune cells and target virally infected brain cells. Importantly, although systemic immune responses against viral vectors injected systemically has thought to be deleterious in a number of trials, results from brain gene therapy clinical trials do not support this general conclusion suggesting brain gene therapy may be safer from an immunological standpoint.

"The world does not speak. Only we do."
Richard Rorty, *Contingency, irony, and solidarity,* 1989.

1. INTRODUCTION

Gene therapy is an attractive and promising tool for the treatment for neurological disorders. Viral vectors are ideal gene delivery vehicles to the CNS due to their ability to infect both dividing and non-diving cells and the ability to produce stocks of concentrations high enough to use as doses small enough required for administration to the nervous system. Numerous pre-clinical and clinical experiments have been performed using gene therapy strategies to treat neurological disorders including Parkinson's disease[During *et al.*, 2001; Muramatsu *et al.*, 2002; Wong *et al.*, 2006; Kaplitt *et al.*, 2007], Alzheimer's disease [Levi-Montalcini, 1987; Eriksdotter Jonhagen *et al.*, 1998; Tuszynski *et al.*, 2005], and glioma[Chiocca *et al.*, 2004; Immonen *et al.*, 2004; Ali *et al.*, 2005]. Unfortunately, researchers have often had difficulty translating the success of preclinical animal gene therapy experiments into significant therapeutic outcomes in the clinic. The patient's immune response to the viral vector is a logical culprit for the lack of substantial therapeutic benefits in clinical trails for the treatment of neurological disorders. Here we examine the immune response against gene therapy vectors in the brain. Particular attention is paid to the unique immunological status of the brain. Transgene expression in the CNS mediated by first generation and high capacity, helper dependant adenoviral vectors is explored in the context both the both the innate and adaptive arms of the immune system.

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2. BRAIN IMMUNE REACTIVITY. LOCATION, LOCATION, LOCATION

The type of immune responses that will be induced as a consequence of viral vector delivery into the brain will depend on which of the two main immune compartments will be targeted by the therapeutic viral vectors. Brain ventricles, meninges and choroid plexi contain all cellular, vascular and lymphatic elements of the immune system, as do most other organs in the body. This includes dendritic cells (DC), the major cell type capable of inducing primary T cell responses. DCs are located within the meninges, choroid plexus and cerebrospinal fluid (CSF) of the normal non-inflamed brain [McMenamin, 1999; Pashenkov *et al.*, 2003]. These DCs are strategically located to capture foreign or self antigens. From their location throughout the brain ventricular system they can migrate to deep cervical lymph nodes (CLN), the primary lymph nodes draining the brain and CSF [Cserr *et al.*, 1992]. The second brain immune compartment, the brain parenchyma itself, is devoid of DCs, typical lymphatic vessels, and is separated from the general circulation by the

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blood brain barrier formed by tight intercellular endothelial junctions[Bechmann *et al.*, 2007]. A large number of molecular mechanisms serve to dampen immune reactivity in the brain parenchyma itself.

What are the immune consequences of making antigens available to either of the brain immune compartments? Injection of a particulate antigen or infectious agent (e.g. live influenza virus, BCG, non-replicative adenoviral vectors) only into the brain parenchyma causes innate inflammatory responses, but does not trigger systemic adaptive immune responses[Matyszak *et al.*, 1996; Stevenson *et al.*, 1997b; Cartmell *et al.*, 1999; Lowenstein, 2002]; i.e. the systemic immune system remains ignorant of the delivery of antigens into the brain. However, injection of the same type of antigen into the ventricular system, will stimulate an innate inflammatory and a systemic adaptive immune response [Matyszak *et al.*, 1996; Stevenson *et al.*, 1997a; Matyszak, 1998]. Different rules apply upon the injection of a soluble diffusible ligand. Thus, injection of a soluble diffusible antigen (e.g. OVA) into either compartment, does induce a systemic B cell response [Knopf *et al.*, 1998]. The reason for this is that independent of its site of delivery, the antigen will diffuse to reach the CLN and stimulate a systemic humoral immune response. Particulate antigens carefully delivered to the brain parenchyma fail to do so because they cannot diffuse out into the ventricular system to reach the CLN.

The differential distribution of DCs is very important to organize these differing brain immune responses. Throughout the body, DCs localize predominantly to lymphoid tissue, where they take up antigen and mature to potent antigen presenting cells (APC). Alternatively they acquire antigen at inflamed sites from where they migrate to secondary lymphoid organs to activate naïve T cells[Caux *et al.*, 2000; Leon *et al.*, 2007].

Could DCs follow similar pathways during brain infections, or inflammatory states caused by various agents including gene therapy vectors? DC could take up antigens in the brain ventricles, mature, and migrate to CLN. Alternatively, antigens could drain directly into deep CLN by diffusion. A possible reason underlying the different immune responses upon antigen delivery to either brain ventricles or parenchyma may be due to inability of particulate antigen to move from the brain parenchyma to the CLN, using either diffusion or cellular transport. Thus, particulate antigens injected into the brain parenchyma, though they may cause inflammation, are never transported to the lymph nodes to prime a systemic immune response, and thus, the systemic immune system remains ignorant of their presence. Soluble antigens on the other hand can and will diffuse from the brain to the ventricles, reach the lymph nodes, and stimulate systemic adaptive immune responses.

Independently of antigen transport routes to the lymph nodes, naïve T cells are primed in the CLN, expand, exit the lymph nodes, and traffic to the site of insult, where they will exert their effector function upon antigen re-encounter. Thus, although DCs can enter the CNS parenchyma during inflammation to sustain T cell function, initial T cell activation preceding disease onset is most likely to occur in the CLN. During chronic inflammation, however, brain infiltrating DC have been proposed to be able to present antigen to naïve T cells[McMahon *et al.*, 2005].

3. IMMUNE RESPONSES AGAINST VIRAL VECTORS USED IN GENE THERAPY

a. Innate Immune Responses. *Kickstarting the Defense*

As many gene therapy strategies employ viral vector technology, the immunogenicity of the gene therapy must be thoroughly evaluated and characterized. Delivery of viral vectors into the CNS induces acute inflammation including microglial activation, macrophage recruitment, and antigen-non specific T cell infiltration. This innate immune response is dose-dependant and independent of the immune status of the particular compartment of the CNS to which the viral vectors are delivered. As recombinant viral vectors are packaged into identical capsids as wild type viruses, the two are indistinguishable by the immune system. In most cases the interaction between viral capsid proteins and specific innate immune receptors, such as Toll receptors, mediates the initial inflammatory response Due to the compartmentalization of the brain's immune system, injection of viral vectors into the immune-privileged brain parenchyma stimulates innate inflammatory responses without necessarily inducing a linked systemic adaptive immune response.

In a detailed dose response study, we examined the short term (3 days) and medium-to long term (30 days) inflammatory consequences of injecting increasing doses of adenoviral vectors delivered in small volumes directly into the mouse striatum[Thomas *et al.*, 2001a]. A limited dose (small volume and low dose) of an adenoviral vector delivered directly into the brain parenchyma will not stimulate the systemic adaptive immune response. The limited, if any, availability of vector antigens to the general systemic circulation and lymphoid organs is thought to contribute to this systemic immune ignorance, and thus, lack of priming of an adaptive anti-vector immune response.

Vector doses ranging from 10^6 to 10^9 infectious units were injected directly into the striatum. Both transgene expression and cellular inflammation, including microglia activation, macrophage infiltration, antigen-non specific T cell recruitment, were evaluated at three and thirty days after vector administration. β-galactosidase expression was detected a early as 3 days post vector injection in brains injected with 10^6 infectious units. While expression levels increased with escalating doses, a plateaue was reached following administration of 10^8 infectious units of vector. In addition, cytotoxicity also increased in parallel with increasing doses of vectors injected. Minimal local cytotoxicity was observed at doses below 10^8 iu, but immuno-reactivity for the astrocyte marker GFAP and the neuronal marker NeuN suggested a substantial loss of astrocytes and neurons following the injection of 10^9 iu. Importantly, administration of high doses of heat inactivated adenoviral vectors ($1x10^9$ iu) failed to cause any significant inflammation and/or leukocyte infiltration into the brain. These data demonstrates that acute toxicity is indeed caused by intact viral particles, but not the viral proteins or vector genomes[Thomas *et al.*, 2001a].

Importantly, when studied for medium-to long term periods of time (i.e. 30 days post intracranial vector injection) doses of vectors that elicited an <u>increased</u> inflammatory response consisting of increased markers for monocytes and lymphocytes at 3 days, had a corresponding <u>elimination</u> of

transgene expression at 30 days; i.e., reduced transgene expression at doses of 10^8, and complete abrogation of transgene expression following the injection of 10^9 iu. However, stable transgene expression at 30 days was observed at the doses of 10^6 and 10^7.

TUNEL staining revealed an increase of apoptotic cells demonstrating cytotoxicity was caused by the acute inflammatory induced cell death resulting from the dose of 10^9 infectious units. *Thomas et al.* detected increases in persistent brain inflammation and activation of microglia, and continued presence of monocytes and leukocytes corresponding to an increase in the brain cell loss. Acute cytotoxicity was directly correlated with the long term persistence of macrophages, $CD8^+$ T cells, and increased expression of MHC-I in the CNS. There was also a positive correlation between short-term and medium-to long term (>30 days) brain cellular inflammation and long term loss of transgene expression from adenoviral vectors. Inversely, when brains injected with noncytotoxic doses (i.e. below $1x10^8$) were examined 30 days later, any initial inflammatory mononuclear cell infiltration and microglia activation had completely resolved. These data indicate that the acute innate inflammatory response caused by intracranial injection of adenoviral vectors in naïve animals is dose-dependent, transient, and self-limiting. Moreover, doses less than $1x10^8$ provide therapeutically accepted levels of transgene expression without any long term inflammation, monocyte recruitment, or cytotoxicity.

In a follow-up work, we demonstrated that interferon regulated, and chemokine mRNAs were not unregulated[Zirger *et al.*, 2006] at the non-cytotoxic doses established in the work of Thomas *et al.*[Thomas *et al.*, 2001a] Also using a dose-response regime of increasing doses of adenovirus from 10^5 to 10^8, *Zirger et al* only observed an increase in αβ-interferon-regulated genes such as OAS, IRF-1, and PKR; and chemokines, such as RANTES, MCP-1, and IFN-γ-inducible protein 10, were only significantly increased at the dose of 10^8 infectious units, thus, above the threshold established for activation of local microglia, and recruitment of circulating mononuclear cells, established by Thomas *et al.*[Thomas *et al.*, 2001a] Moreover production of mRNAs for αβ-interferon-regulated genes and chemokines was transient with expression of most mRNAs returning to baseline by 7 days post-injection into the brain. This indicates that innate inflammatory responses to adenovirus (i.e. increase in expression of interferon-inducible genes, and chemokine genes) are dose-dependent. The injection of adenoviral vectors above a particular threshold increases expression of chemokines and induces local cytotoxicity. Once this inflammatory threshold is crossed, long-term, potentially chronic (i.e., 6 months) brain inflammation can be expected.[Dewey *et al.*, 1999]

b. Thresholds: *A Guide to the Administration of Safe Gene Therapy*

The inflammatory threshold to intracranial delivery of adenoviral vectors injected into the brain is 1 x 10^8 iu; once this threshold is crossed, increased expression of interferon-inducible and chemokine-encoding genes, the activation of local microglia, and the recruitment of circulating monocytes and lymphocytes is observed. Injections of vector doses below this threshold do not induce any innate increased recruitment of inflammatory cells to the brain, do not cause glial or neuronal toxicity, do not cause an increase in expression of interferon-regulated genes or chemokine genes, and achieve long term transgene expression of at least up to one year (Barcia *et al.* In Press). The consequences of this work demonstrate the necessity to consider the dose-dependent increased inflammatory gene expression or recruitment of inflammatory cells caused by adenoviral vectors when planning pre-clinical and/or clinical trials.

These studies highlight the importance of using high quality, well characterized viral vectors. Errors in the titration of these vectors and mistakes in the calculation of the amount of vectors administered will have serious, deleterious consequences for long term therapeutic transgene expression in the brain. Adenoviral vectors are very effective as therapeutic vehicles for gene therapy into the brain, *but only at the right dose*.

c. Adaptive Immune Responses Against Adenoviruses Injected Into The Brain: *Calling in Reinforcements*

As a result of the compartmentalization of the brain immune system, careful injection into the brain of non-replicating viral vectors at doses below the established immunological threshold provides long term therapeutic transgene expression and achieves therapeutically effective transgene expression in experimental models of neurological disorders. However, if the viral vectors escape to the peripheral organs or the lymphatic drainage, a systemic adaptive anti-adenoviral immune response mediates an almost complete elimination of transgene expression from the brain. T cells are the main cells responsible for eliminating transgene expression from the brain with very high efficiency. T cells are able to recognize as little as $1x10^3$ iu of vector injected into the brain, an equivalent of 1000 transduced cells.[Barcia *et al.*, 2006a]

If animals are injected with adenovirus intracranially and then immunized systemically against adenovirus within 30–60 days, transgene expression in the brain will be reduced by more 50% (Fig. **1**). In studies by Barcia *et al.,* our group demonstrated that an adaptive immune response was able to eliminate transgene expression from as little as 1000 infectious units injected into the brain.[Barcia *et al.*, 2006a] For these experiments, a dose escalation of adenovirus was injected into the brain of naïve animals, followed by a systemic immunization against adenovirus. The resultant systemic immune response was able to eliminate transgene expression at doses from 10^7 to as low as 10^3 infectious units injected into the brain. The high efficiency by which the adaptive immune system eliminates transgene expression from the brain is highlighted by the detection and elimination of as little as 1000 infected cells in the brain.

Further experiments by Barcia et al[Barcia *et al.*, 2006b], and Zirger *et al.* (in preparation), have shown that immune mediated elimination of transgene expression from the brain is dependant on the presence of $CD8^+$ T cells and $CD4^+$ T cells. We have also shown that the elimination of transgene expression was independent of the promoter used to drive transgene expression; whether viral, housekeeping, or cell type specific promoters are used to drive transgene expres-

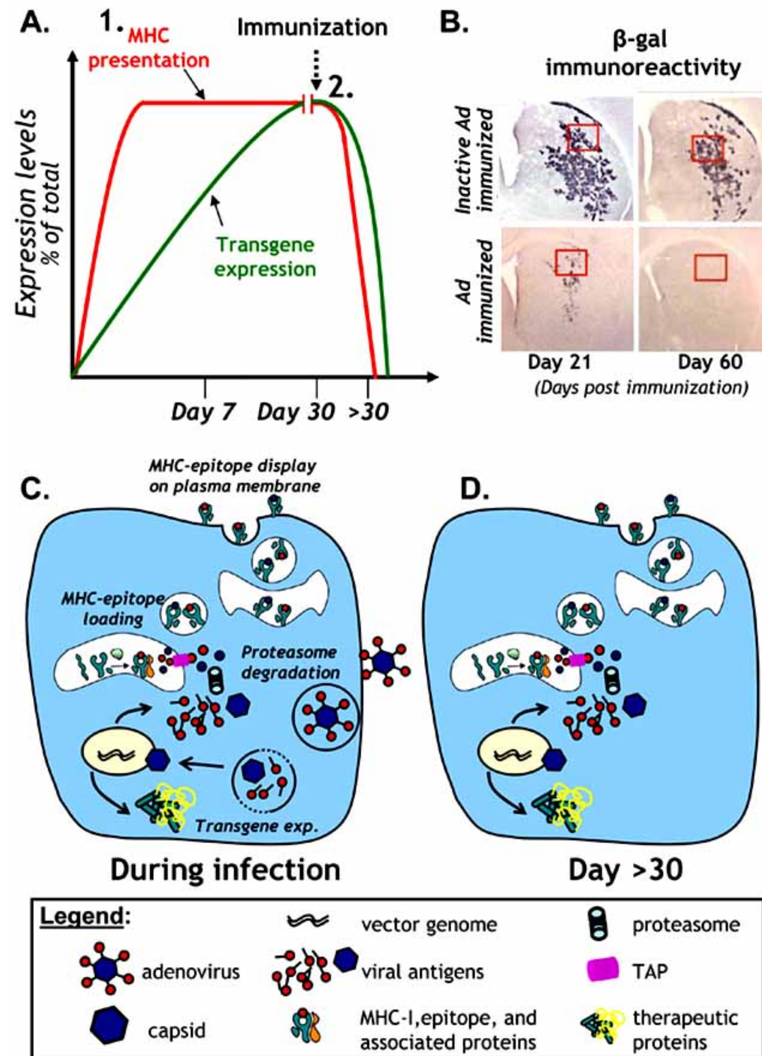

Fig. (1). Anti-adenoviral immune responses completely eliminate transgene expression from first generation adenoviral vectors. A. The levels of transgene expression and MHC presentation of viral epitopes in animals injected with a first generation adenoviral vector into the CNS are illustrated. MHC-I presentation of viral epitopes peaks earlier due to intracellular degredation and presentation of capsid derived epitopes. Transgene expression (and consequent MHC antigen presentation) are completely abrogated following systemic immunization with adenovirus. **B.** Immunoreactivity for β-galactosidase illustrates the effects of a systemic immune response against adenovirus on transgene expression from first generation adenoviral vectors in the brain of rats immunized against inactivated adenovirus (top panels) or adenovirus (bottom panels). Short term (21 days post immunization, left panels) and medium term (60 days post immunization, right panels) expression of β-gal is shown. Note the sharp reduction of β-gal immunoreactivity in immunized animals compared to controls animals immunized with inactivated adenovirus. Sixty days later, transgene expression is completely eliminated in immunized animals while transgene expression is sustained in control immunized animals. **C.** A schematic of first generation vector infection, uncoating, nuclear transduction, production of transgene and presentation of viral antigenic epitopes on MHC-I is shown shortly after and during vector infection. **D.** A schematic of the same cells is shown >30 days later. Note the continued expression of viral proteins from the first generation adenoviral vector genomes and their presentation by MHC molecules on the cell surface.

sion, the immune system is able to eliminate transgene expression from all of these promoters[Barcia *et al.*, 2006a].

d. High Capacity, Helper Dependent Adenoviruses. *A Radical Makeover*

The adverse immune responses would appear to mount an insurmountable block to stable, sustained, therapeutic gene therapy. Having identified the immune challenges, and in order to overcome these obstacles a new generation of viral vectors, known as high capacity, helper dependent adenoviruses (HC-Ad) have been recently developed to evade detection the immune system. These allow the insertion of up to 30 – 34 Kb of transgenic sequences but most importantly, the genomes of these vectors do not encode any viral

proteins. Therefore, the HC-Ad genomes do not produce any adenoviral proteins *in situ* that could be recognized as antigenic epitopes by the immune system (Fig. **2**). A series of papers by Thomas *et al.,* [Thomas *et al.*, 2000] Thomas *et al.,* [Thomas *et al.*, 2001a] Xiong et al,[Xiong *et al.*, 2006] Barcia et al, (Molecular Therapy, In Press), O'Neal *et al.,* [O'Neal *et al.*, 2000] Mian *et al.,* [Mian *et al.*, 2005] Parks *et al.,* [Parks *et al.*, 1999] Maione *et al.,* [Maione *et al.*, 2001] and others have now demonstrated that transgene expression from these viruses remained stable, and was shown to persist for up to one year (Fig. **2B**) Barcia et al, (Molecular Therapy, In Press), even in the presence of pre-existing immune response against adenovirus. Therefore, these vectors could be used even in human patients that have been pre-exposed to adenovirus before being subjected to gene therapy, as would be the case in the vast majority of human patients undergoing an experimental gene therapy using an adenoviral gene delivery system[Thomas *et al.*, 2000; Thomas *et al.*, 2001a; Xiong *et al.*, 2006].

Before HC-Ad vectors infect their target cells in the brain, the viral capsid of these vectors could, of course, be neutralized by anti-adenovirus antibodies. However, the adaptive arm of the immune system, the T cells, would have a very short period of time during which they can recognize cells that have been infected with HC-Ad vectors. Capsid proteins could be transiently presented on MHC Class1 molecules. However, such proteins would only be provided by the capsid directly administered, because the genome of these vectors does not encode for any viral proteins. Once these input virion proteins from the viral capsid have been metabolized, the HC-Ad vector effectively becomes immune to the antiviral T cells. Once the genome of these viruses has reached the nucleus, HC-Ad vectors are completely immune to the adaptive arm of the immune system (Fig. **2D**). Therefore, further engineering of these vectors shows that adenoviruses are effective vehicles for long term therapeutic transgene expression in the brain.

e. Administering Gene Therapy to Pre-Immunized Animals: A Clinically Relevant Paradigm

To mimic the anti-adenovirus immunization status of patients unrolled in clinical trials using an adenoviral vectors, Thomas *et al.* and Barcia *et al.* performed experiments to assess the longevity of transgene expression in animals that had been *pre-immunized* against adenoviral *before* intracranial delivery of Ad vectors[Thomas *et al.*, 2001b] and (Barcia *et al.,* In press). This experimental paradigm was used to assess intracranial transgene expression in the presence of a systemic anti-adenoviral immune response in rats for up to 60 days post intracranial delivery [Thomas *et al.*, 2001b] and also in mice for up to one year after intracranial delivery (Barcia *et al.,* In press) (Fig. **3**). In both species intracranial transgene expression mediated by first generation adenoviral vectors was nearly abolished (mouse) or totally abolished (rats) within 60 days in animals immunized systemically compared to control, saline immunized animals (**Fig 3B**). As expected, HC-Ad elicited sustained intracranial transgene expression even in the presence of an ant-adenoviral immune response in both rats and mice. We detected high levels of HC-Ad mediated transgene expression in the brains of animals at the longest timepoint tested (i.e 60 days in rats and 365 days in mice) (**Fig 3C**). In fact the levels of HC-Ad mediated transgene expression were indistinguishable between mice immunized with adenovirus or saline alone for all timepoints tested between 14 to 365 days (Barcia *et al.,* In Press). These data further support implementing the HC-Ad vector platform into upcoming clinical trials for neurological disorders.

We also performed pre-immunization studies to examine the effects of a systemic immune response against the vector encoded transgene. To do so, Xiong *et al.*. [Xiong *et al.*, 2007] immunized naïve mice with a systemic administration of a mammalian expression plasmid containing the transgene β-galactosidase or saline alone. Animals then received an intracranial administration of an HC-Ad vector encoding β-gal and the brains were analyzed for β-gal immunoreactivity seven weeks later (Fig. **3C**). Quantitative stereology revealed a statistically significant decrease in β-gal immunoreactivity in animals pre-immunized against β-gal when compared to mice immunized with saline alone (Fig. **3D**). These data and data from other groups[Molnar-Kimber *et al.*, 1998; Favre *et al.*, 2002; Lena *et al.*, 2005; Di Domenico *et al.*, 2006; Shi *et al.*, 2006] suggest that transgene-specific immune responses must also be taken into consideration when planning and implementing a gene therapy clinical trial. Whether the mechanisms that reduce transgene expression in the brain and peripheral organs are the same remains to be determined.

f. Immune Responses Against AAV Vectors Injected into the Brain

Adeno-associated virus (AAV) is a small single-stranded DNA virus that was originally discovered as a contaminant in cell lines used to study adenovirus [Muzyczka *et al.*, 2001]. Similar to HC-Ad, all the viral encoding genes have been removed from the recombinant AAV (rAAV) and replaced with a transgene and transcriptional elements inserted between two ITRs [Muzyczka *et al.*, 2001]. Wild-type AAV is not associated with any known human disease and requires helper functions from Ad or other cytotoxic viruses in order to replicate and infect new host cells [Muzyczka *et al.*, 2001]. Also, like Ad5, the majority of the human population has been exposed to the most common AAV serotype (AAV2) resulting in circulating neutralizing antibodies [Blacklow *et al.*, 1968; Blacklow *et al.*, 1971; Chirmule *et al.*, 1999; Erles *et al.*, 1999]. Immune responses to rAAV2 have been reported in the peripheral immune system, where competent DCs and lymph drainage exists[Zaiss *et al.*, 2005; Mingozzi *et al.*, 2007].

rAAV has been used extensively in the brain [Mandel *et al.*, 2006] and is currently in several clinical trials for neurological disorders [Mandel *et al.*, 2004; Kaplitt *et al.*, 2007]. Due to the immune compartmentalization of the brain and lack of an adaptive immune response as discussed above[Matyszak *et al.*, 1996; Stevenson *et al.*, 1997b; Cartmell *et al.*, 1999; Lowenstein, 2002], intra-parenchymal brain administration of rAAV has been thought to induce little immunogenicity especially when injected once in the parenchyma of naïve animals [Mandel *et al.*, 1998; Lo *et al.*, 1999; Mastakov *et al.*, 2002; Peden *et al.*, 2004].

As with adenovirus, transient, innate inflammatory responses to highly purified rAAV also occur when higher

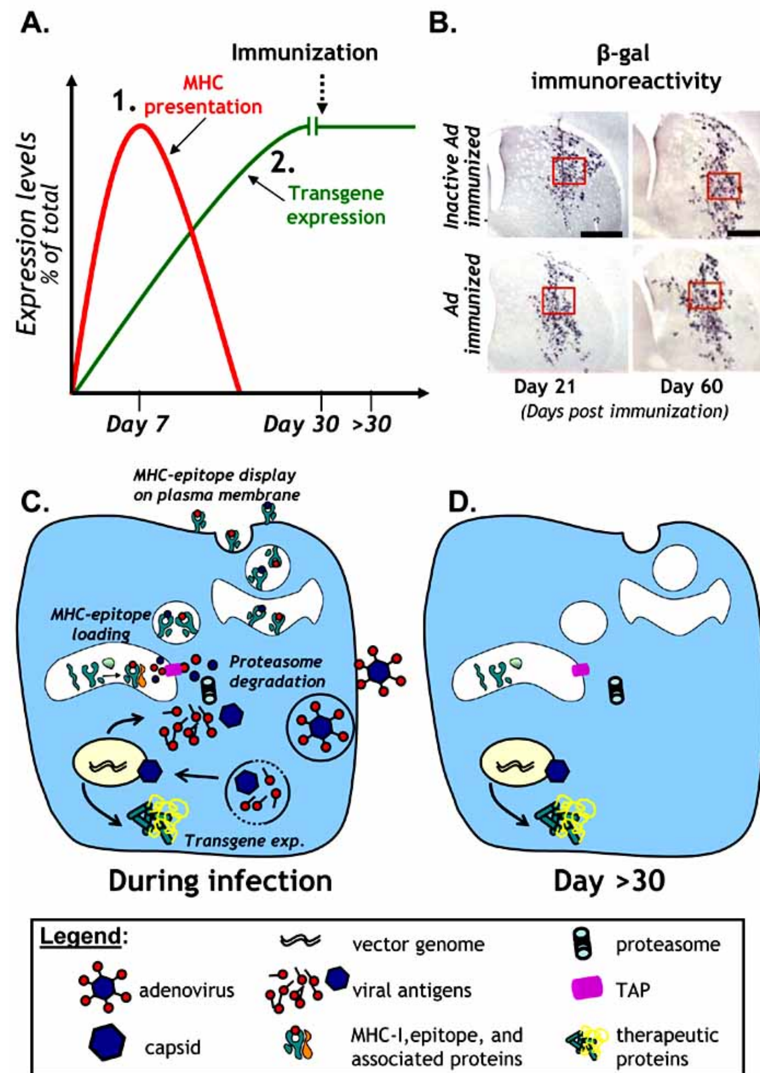

Fig. (2). Anti-adenoviral immune responses are incapable of eliminating transgene expression from HC-Ad vectors. A. The levels of transgene expression and MHC presentation of viral epitopes in animals injected with a HC-Ad vector into the CNS are illustrated. MHC presentation of viral protein is short-term because only capsid derived epitopes can be presented during capsid degredation. Transgene expression in the brain is sustained, even after systemic immunization against adenovirus. **B.** Immunoreactivity for β-galactosidase illustrates the failure of a systemic immune response against adenovirus to eliminate transgene expression from HC-Ad vectors in the brain of rats immunized against adenovirus (top panels) or saline alone (bottom panels). Short term (21 days post immunization, left panels) and medium-long term (60 days post immunization, right panels) expression of β-gal is shown. Note the sustained expression of β-gal in both Ad-immunized animals and controls animals immunized with saline alone at 14 and 60 days immunization. **C.** A schematic of HC-Ad vector infection, uncoating, nuclear transduction, production of transgene and presentation of viral antigenic epitopes on MHC-I is shown after vector infection. **D.** A schematic of the same cells is shown >30 days later. Note the absence of expression of viral proteins from the HC-Ad vector and the lack of presentation of viral epitopes by MHC molecules on the cell surface.

doses are administered into the naïve brain parenchyma [Reimsnider *et al.*, 2007]. The discrepancy regarding the innate inflammatory responses between earlier reports from our lab [Mandel *et al.*, 1998] and our recent data [Reimsnider *et al.*, 2007] is most likely due to the increased titers of rAAV injected. Thus, although no formal study of a dose-dependant innate immune response to rAAV have been undertaken, there was little or no detectable innate immune response when injecting lower titers of rAAV2 vectors (2 x 10^8 particles [Mandel *et al.*, 1998], 4 x 10^8 [Peden *et al.*, 2004]) 4 weeks after intrastriatal transduction. However when injecting higher titers rAAV2 vectors (≈ 4 x 10^{10} particles) [Reimsnider *et al.*, 2007] a significant transient GFAP (glial fibrillary acidic protein) response was observed. Thus, the observation of an increased striatal inflammatory response to increasing titers of rAAV2 could be considered similar to the observation that intrastriatal injections of increasing titers of adenoviral vectors also leads to an innate

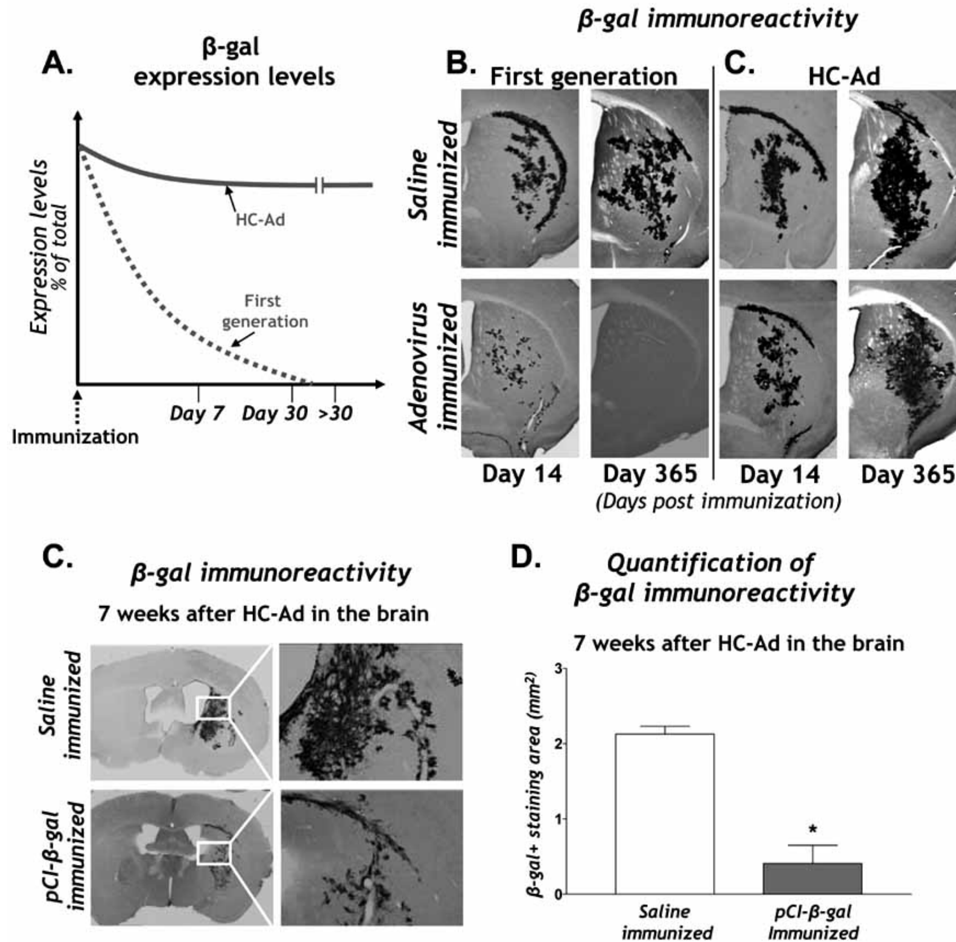

Fig. (3). Comparison of pre-existing responses against first generation adenovirus, high capacity adenovirus, and transgene. A. The levels of intracranial transgene expression in the presence of a pre-existing systemic anti-adenovirus immune response are shown. Note the sharp reduction of transgene expression following intracranial delivery of first generation adenovirus compared to HC-Ad transgene expression. **B.** First generation adenovirus and **C.** HC-Ad mediated transgene expression in the mouse brain is visualized by β-gal immunoreactivity at 21 days and one year post intracranial vector delivery. Note the sustained expression of β-gal from both vectors in control immunized animals and persistence of expression in animals injected with HC-Ad even at one year post vector injection compared to the complete ablation of transgene expression in animals injected with first generation adenoviral vectors. **C.** β-gal immunoreactivity in the brain of animals pre-immunized with saline (top panels) or with β-gal (bottom panels) is shown seven weeks post intracranial vector delivery. Right panels depict high magnification images of immuoreactive area. **D.** Quantitative stereology of β-gal immunocytochemistry reveals a statistically significant decrease in β-gal immunoreactivity in animals pre-immunized with β-gal when compared to animals immunized with saline alone.

immune response once a threshold Ad titer is reached [Thomas *et al.*, 2001a].

In addition to single injections of rAAV2 into naïve rats, re-administration of rAAV2 vectors in the opposite hemisphere either 2 or 4 weeks apart induces a significantly greater inflammatory response in the second injection site [Mastakov *et al.*, 2002; Peden *et al.*, 2004] (Fig. **4a**). Mastakov et al. [Mastakov *et al.*, 2002] also reported reduced expression of the rAAV2-mediated luciferase transgene in the second injection site whereas Peden et al. [Peden *et al.*, 2004], did not observe reduced transgene expression when using human glial cell line-derived neurotrophic factor (GDNF) as the transgene [Peden *et al.*, 2004]. However, we have subsequently replicated the loss of rAAV2-mediated transgene expression in the second injection site when using GFP as the transgene (Peden and Mandel, unpublished observations).

Upon first consideration, a significant rAAV-induced re-administration immune response in the brain is difficult to explain since no viral genes are expressed after rAAV trans-

duction due to the lack of genes encoding viral proteins. However, rAAV2 vectors have been reported to uncoat slowly, thus potentially allowing processed capsid peptides to be presented via MHC-I [Lowenstein, 2004; Thomas *et al.*, 2004; Herzog, 2007] (Fig. **2C**). Antigen presentation of AAV2 capsid peptides as the etiology of an increased inflammatory response in the second injection site is supported by increased MHC-I expression that we observe in the second injection site after intrastriatal delivery of rAAV2 vectors [Peden *et al.*, 2004](Fig. **4b**). The other possibility besides the extended presentation of AAV2 capsid peptides is a transgene specific inflammatory response. However, when we re-administer a different serotype, rAAV5 expressing GFP in the second injection site, no enhanced inflammation or reduction of rAAV-transduced GFP+ neurons was observed (Reimsnider, Manfredsson and Mandel, unpublished observations). Further supporting the hypothesis of extended presentation of AAV2 capsid peptides , Mastakov *et al*. reported that transgene expression was unaffected following re-administration of rAAV2 three months after the first intrastriatal injection [Mastakov *et al.*, 2002]. We have also recently replicated the phenomenon that a longer delay between rAAV2 administrations allows high levels of transgene expression following re-administration of the vector (Reimsnider, Manfredsson, and Mandel unpublished observations). Finally, we have shown that acute re-administration of rAAV5 vectors, which uncoat more rapidly than rAAV2 capsids, does not result in increased inflammation or transgene reduction in the second injection site.

All of the foregoing experiments were carried out in animals that were naïve to wt-AAV since wt-AAV does not ordinarily infect rodents. However, because a significant portion of the human population has been exposed to wt-AAV [Blacklow *et al.*, 1968; Blacklow *et al.*, 1971; Chirmule *et al.*, 1999; Erles *et al.*, 1999], it is important to study any potential immune response against rAAV in the CNS of pre-immunized animals. Thus, two studies demonstrated that intrastriatal rAAV2-mediated transduction was completely blocked by systemic pre-immunization against wt-AAV2 [Peden *et al.*, 2004] or against rAAV2 [Sanftner *et al.*, 2004]. In both studies, high levels of circulating anti-AAV2 neutralizing antibodies were observed and these antibodies were specific for AAV2 since rAAV5-mediated transduction was unaffected [Peden *et al.*, 2004]. These studies clearly establish that circulating neutralizing antibodies can affect intracerebral rAAV-mediated transduction. Moreover, when rAAV2 pre-immunized rats underwent an rAAV2 re-administration paradigm, a qualitatively greater immune response was seen in the second injection site as compared to the second injection site in non-immunized rats [Peden *et al.*, 2004]. These data suggest that the adaptive arm of the immune system can be primed by intracerebral rAAV2 administration. This consideration is especially important because memory T cell responses to AAV1, AAV2, and AAV8 have been demonstrated in healthy individuals[Mingozzi *et al.*, 2007]. Moreover, as mentioned above, it has been proposed that parencyhmal inflammation can lead to the infiltration of the brain by dendritic cells that may be able to present antigen to naïve T cells [McMahon *et al.*, 2005]. Therefore, although the naïve brain is relatively immunoprivileged, especially with regard to its incapacity to prime T cells, CD8+ T cells can be found in the brain in pre-immunized animals inoculated with intrastriatal rAAV2 [Peden *et al.*, 2004].

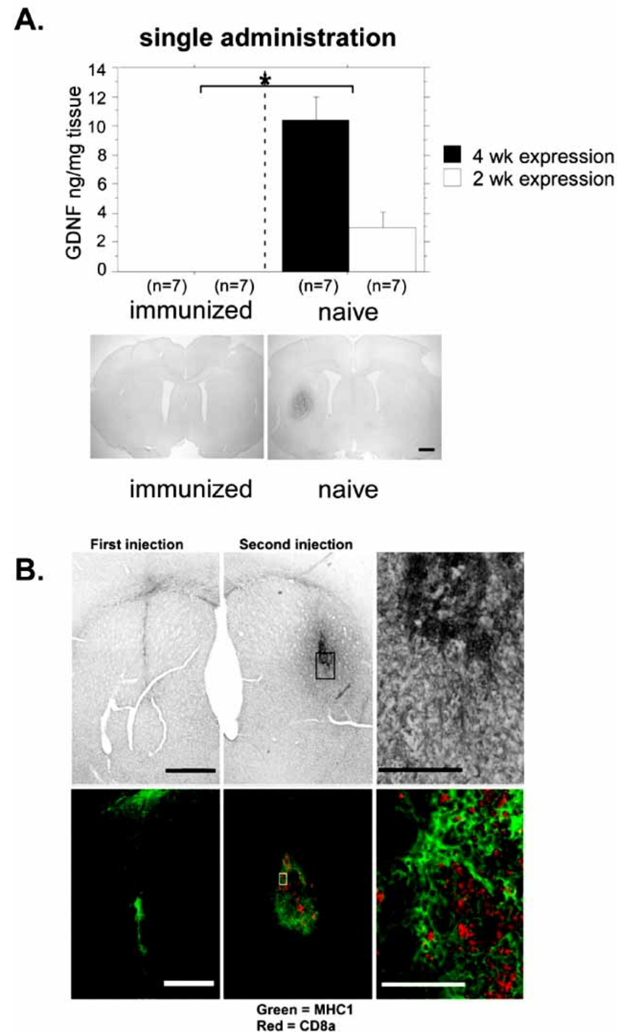

Fig. (4). Pre-existing responses against AAV vectors. A. Intrastriatal GDNF expression as determined by ELISA (top panels) and immunohistochemistry (bottom panels). Immunization with wt AAV2 completely blocked GDNF expression at both the 2- and 4-week time points following rAAV2-GDNF striatal transduction.as evidenced the striatum by both ELISA and ICC when compared to naïve animals. Injection of rAAV2 in the right striatum completely blocked GDNF expression when AAV2-GDNF was readministered in the left striatum (bottom left panel). The bottom right panels shows a representative section from a naive animal 4 weeks after rAAV2-GDNF injection in right striatum, with no further treatment. **B.** Striatal sections were immunostained with for MHC-I (green) and CD8α (red) in animals injected with rAAV. Note the increase of CD8α immunoreactivity in the brains of animals that received a second injection (middle panel) as compared to those animals only receiving a single injection (left panel). The right panel is a higher magnification image of the middle panel.

Thus, utilizing careful *in vitro* assays to detect AAV capsid pre-exisiting CD8+ T cell responses to proteins, such as those described by Mingozzi et al. [Mingozzi *et al.*, 2007] prior to rAAV administration, might be useful in rAAV clinical trials for neurological disorders. This may be especially important when using AAV vectors. Because of their smaller size they may be able to diffuse out of the brain, even following careful delivery into the brain parenchyma; this does not occur following the injection of the larger adenoviral vectors.

In a recent Phase I clinical trial for Parkinson's Disease using a 50μL injection of AAV2 vector expressing glutamic acid decarboxlyase (GAD) into the subthalamic nucleus, there was no change in the levels of pre-existing anti-AAV2 circulating antibodies and/or neutralizing at any of the time points tested following treatment (1, 3, 6, and 12 months) compared to baseline levels. The work published by Kaplitt and co-workers suggests that careful administration of small volumes of AAV2 into the brain did not induce a measurable systemic antibody immune response against AAV2. Moreover, neutralizing antibodies against the therapeutic protein were not detected for up to one year following administration of the viral vector[Kaplitt *et al.*, 2007]. The existence of T cell responses, much more difficult to detect, but potentially more deleterious remain to be explored in gene therapy brain clinical trials. In a clinical trial for Canavan disease using 900μL of AAV2 expressing aspartocylase (ASPA) split amongst six injection sites, investigators found low to moderately high levels of neutralizing antibodies to AAV2 in 3 out of 10 patients compared to pre-gene therapy levels [McPhee *et al.*, 2006]. The differences in the neutralizing antibody responses between these two clinical trials could be attributed to differences in intracerebral injection volumes, anatomical site of injection, and the underlying brain pathology. These issues remain to be monitored in future ongoing studies.

In summary, in contrast to Ad, there have been few studies of the immune response after rAAV administration in the brain. However, the studies that exist indicate that there can be rAAV-induced immune reactions especially when using high titer viruses [Reimsnider *et al.*, 2007]. The existence of a dose threshold above which inflammation is caused indicates the need to carefully monitor the dose and purity of the vector to keep within safe, non-toxic parameters. Thus, it is clear that more careful analysis of rAAV-mediated immune responses in the brain, similar to the studies that have been carried out with Ad, should be undertaken.

4. WHAT CAUSES LOSS OF TRANSGENE EXPRESSION? *TO BE KILLED, SILENCED, OR COMMIT SUICIDE, THE ULTIMATE EXISTENTIAL QUESTION*

While the case has clearly been made that the adaptive immune response is able to eliminate transgene expression from the brain, the mechanisms by which transgene expression is eliminated is not yet understood. Moreover, the consequences or "the fate" of the cell infected by the viral vector has yet to be definitely elucidated. In theory, two main possibilities could account for the loss of transgene expression. On the one hand, T cells, through the secretion of the effector molecules like IFNγ, could selectively turn off or silence transgene expression from transduced cells of the CNS while maintaining the presence of the vector genome. Alternatively, T cells could be able to eliminate transgene expression by killing transduced cells by cytotoxic pathways. The consequences of the particular mechanism of clearance of virally transfused cells are of paramount important. If T cells are able to silence expression from the viral genome, no anatomical damage is being done. Moreover, it could be possible to restart transgene expression from the quiescent vector genome. However, if cytotoxic T cells eliminate transgene expression by killing transduced cells, the consequences to gene therapy clinical trials would be more severe. So far, work done in various laboratories has been inconclusive on this issue.

Sorting out whether the immune system can kill brain cells directly has been difficult to determine beyond reasonable doubt. From a teleological point of view if would clearly be preferably to protect brain cells from immune-mediated cell death. However, this preconception clearly cannot influence the outcome of neuroimmune interactions. If CTLs could kill brain cells these would need to express MHC-I. Although emphasis has been placed on determining whether brain cells, astrocytes and neurons, express MHC-I, information on whether the full complement of the intracellular machinery needed to load antigenic peptide onto MHC-I is present in brain cells is not known. Thus, even if expression of MHC-I has been shown in some cases, strong evidence that MHC-I on neurons can truly server to bind the TCR of CTLs remains to be uncovered. It is also highly likely that whether the immune system can eliminate brain cells will depend on the antigens expressed by brain cells, which virus they are being expressed from, and the nature of the immune response that was stimulated to detect infected cells in the brain. Dissecting these issues is part of important ongoing investigations by several laboratories.

Some of the limitations in detecting brain cell death relate to the number of infected cells in target brain areas. Because the number of infected cells is low (i.e. generally below 5% for the total amount of brain cells in any particular brain regions) loss of such relatively small percentage of brain cells be very difficult to detect experimentally. Added to this, the brain repairs itself rather effectively through astrocytosis, and dead brain cells are quickly phagocytosed by local microglial cells and possibly incoming macrophages, thereby keeping brain inflammation at a reasonable minimum.

For the long term success of clinically effective neurological gene therapy, the ultimate consequences of the mechanisms of elimination of transgene expression are of crucial importance. If the immune system turns off transgene expression through inhibitory transcriptional mechanisms, no permanent anatomical damage will ensue. However, if the immune system kills transduced cells, the underlying disease could worsen. Therefore, the detailed mechanisms by which the immune system abolishes transgene expression from transduced brain cells needs to be determined in order to develop alternative manners to escape, manipulate, or hide, from the immune system. A potential solution would be short-term immunosuppression while the viral capsid epitopes are displayed on MHC-I during vector uncoating

[Herzog *et al.*, 2001; Lowenstein, 2005; Mingozzi *et al.*, 2007]. As discussed above (Figs. **1** and **2**), the duration of viral capsid antigen presentation on MHC-I, and consequent necessary immunosuppression, would be determined by the half-life of the viral capsid proteins (i.e first generation Ads, HC-Ads or AAV vectors).

5. THE CELLULAR SUBSTRATE OF BRAIN IMMUNE RESPONSES: THE IMMUNOLOGICAL SYNAPSES

Recent advances in optical imaging and molecular methods allow to study immune reactions at the single cell level. Thus, the biology of immune responses can now be studied at level of single cell interactions *in vivo*, in addition to analysis of the function of large populations of immune cells studied using methods such as CTL and ELISPOT assays. Over the last ten years a structural rearrangement of proteins at the interfaces of interacting immune cells has been characterized as immunological synapses. Immunological synapses form between T cells and various types of antigen presenting cells, and consist of a reorganization of membrane proteins (i.e., intercellular adhesion molecules [e.g. ICAM-1], TCR), intracellular TCR downstream signaling tyrosine kinases, as well as cytoskeletal structures, and intracellular organelles of the secretory pathway of the T cells [Monks *et al.*, 1998; Grakoui *et al.*, 1999; Dustin *et al.*, 2000; Lee *et al.*, 2002; Huppa *et al.*, 2003; Friedl *et al.*, 2005; Huse *et al.*, 2006; Cemerski *et al.*, 2007]. The formation of immunological synapses is thought to represent the morphological expression of vectorial immune exchanges between T cells and antigen presenting cells.

A canonical structure, described as the mature immunological synapse has been described as consisting of the following arrangement: a peripheral supramolecular activation cluster (pSMAC), consists of a ring of adhesion molecules that anchor the membrane of the T cell to the membrane of the APC, while a central-SMAC (cSMAC), consists of a higher concentration of TCR and signaling molecules. Immunological synapses have been described for both $CD4^+$ and $CD8^+$T cells, and NK cells in contact with various types of APCs, e.g. dendritic cells, B cells, or target cells. Most studies on immunological synapses have studied the interactions between T and APCs *in vitro*, or the interaction of T cells with artificial bilayer membranes.

Most recently, the existence of mature type immunological synapses has also been described in the brain *in vivo* during an immune-mediated elimination of adenovirally infected cells from infected astrocytes. Anti-adenoviral $CD8^+$ T cells infiltrate the brain and form Kupfer-type mature immunological synapses with astrocytes that express MHC-I[Barcia *et al.*, 2006a; Barcia *et al.*, 2006b]. Immunological synapses displayed the typical organization of TCR and LFA-1 into supramolecular activation clusters (SMACS), which constitute the hallmark of immunological synapses originally described by the group of Abraham Kupfer (Fig. **5**).

The characteristics of our experimental model to characterize the cellular and molecular biology of adaptive immune responses against a non-replicating adenoviral gene therapy vector are the following. A first generation adenoviral vector was used to target astrocytes in the rat brain. This virus is replication-defective and thus unable to directly kill infected cells. As the parenchymal CNS infection itself does not induce significant inflammation nor a systemic anti-adenoviral immune response, systemic anti-adenoviral immunization to target the infected brain cells was induced by immunizing systemically with a different Ad vector injected subcutaneously. Systemic anti-adenoviral immunization triggered a systemic anti-adenoviral immune response, which led to brain infiltration of antiviral T cells and brain inflammation. Brain inflammation consisted mainly of an infiltration of the brain parenchyma with CD8 T cells and macrophages that homed in to the area occupied by infected astrocytes, and a perivascular infiltration of CD4 T cells.

The systemic anti-adenoviral immune response resulted in a significant reduction in the number of transduced brain astrocytes; this was accompanied by a reduction in the number of viral genome copy numbers present in the CNS, as would be expected if the lymphocytes were killing infected brain cells. The presence of CD8 T cells within the brain parenchyma suggested the operation of direct cytotoxicity, though no direct evidence for apoptotic astrocytes was obtained. Nevertheless, macrophages containing remains of infected astrocytes were found, indicating that infected cells had been cleared by brain monocyte-derived cells.

We thus proposed that immunological synapses represent the microanatomical substrate underlying CD8 T cell effector functions in the CNS (Fig. **6**) and mediate the clearing of infected astrocytes by CD8 T cells. The importance of these studies is the demonstration that immunological synapse do form indeed *in vivo* in the brain during the clearing of virally infected astrocytes by the adaptive immune response. Their *in vivo* description in the context of an anti-viral immune response highlights their physiological role as the structure underlying neuroimmune interactions *in vivo*. These data further demonstrate that $CD8^+$ T cells are able to directly interact with and eliminate infected astrocytes. If we consider immunological synapses as the substrate of T cell interactions with target cells *in vivo*, we can then use them to help us to dissect whether effector mechanisms are exerted through direct or indirect interactions. For example, the visualization of $CD8^+$ T cells directly interacting with astrocytes to be cleared argues for a direct cytotoxic mechanism underlying the elimination of infected astrocytes; however, if we would only be able to visualize $CD8^+$ T cells interacting with macrophages, we would have to postulate an indirect mechanism to account for the clearing of virally infected astrocytes. The study of the *in vivo* cell biology of effector immune mechanisms in the brain will allow us to dissect the detailed cellular pathways through which the immune system achieves its functions *in vivo*.

6. CONCLUDING REMARKS

The communication between $CD8^+$T cells and resident cells of the CNS is highly sophisticated; yet our understanding of these complex *in vivo* interactions is only now emerging. Major limitations reside in the unique interactions and crosstalk between CNS cells amongst themselves, their fully differentiated state in mature individuals, and their complex three dimensional structures. The network of the brain makes

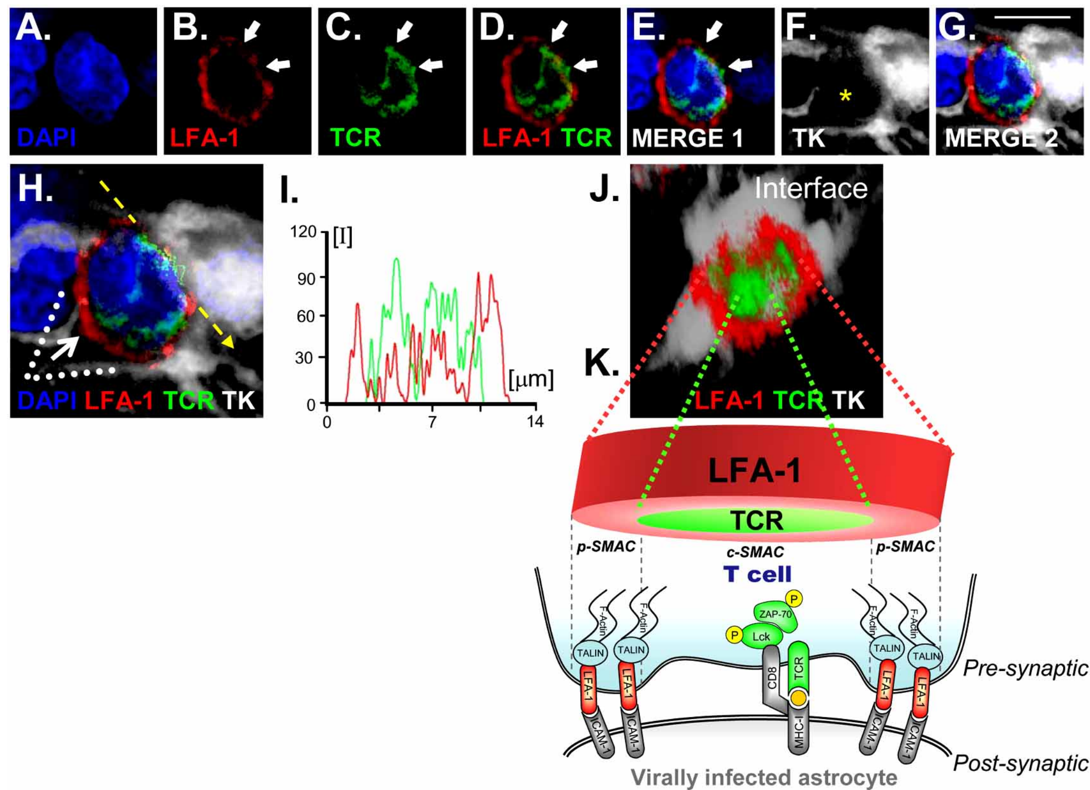

Fig. (5). SMAC formation at immunological synapses *in vivo*, between T cells and infected astrocytes in the brain. Upper panels illustrate confocal images of: DAPI (blue), LFA-1 (red), TCR (green), and the virally infected cell (TK; in white) (Fig. **1A-F**). Scale bars=15 μm. The yellow asterisk in **F** indicates the location of the T cell. Low (**G**) and high (**H**) magnifications of the synapse are illustrated. 3D reconstructed images (**J**) illustrate the characteristic structure of the p-SMAC (outer LFA-1 ring) and c-SMAC (inner TCR cluster) of the immunological synapse. The image shown in **J** was rotated so that the plane of the interface of the immunological synapse (broken arrow in **1H**) could be observed from above (white arrow in **H** shows the angle of vision of the 3D reconstruction in **J**). **I** illustrates the intensity of fluorescence measured at the interface (yellow line in **H**) of the immunological synapse. The graph shows the relative intensity values of fluorescence of LFA-1 (in red) and TCR (in green), showing the expected distribution with more intense LFA-1 staining towards the outside p-SMAC, and stronger TCR in the c-SMAC. **K** is diagrammatic view of a T-cell contacting an infected astrocyte illustrating the localization of molecules involved in the immunological synapse as well as polarized phosphorylated tyrosine kinases. LFA-1 transduces signals to the cytoskeleton through talin, and binds to ICAM-1 on the target cells.

it difficult to extrapolate results obtained from *in vitro* studies using primary cultures from neonatal tissue exposed to lymphocytes removed from whole animals, to events occuring *in vivo*. Although glial cells and neurons are capable of expressing MHC-I *in vitro* and thus acting as targets for *in vitro* stimulated $CD8^+$ T cells, the mode of $CD8^+$ T cell function *in vivo* has largely been deducted from indirect evidence, such as viral clearance, loss of detection of viral genomes, demyelination or tissue atrophy. How T cells eliminate viral infections from the CNS, and whether they could do so by directly contacting infected brain cells, or indirectly through interactions with brain microglial cells remains contested.

How lymphocytes clear viral brain infections, and brain cells transduced with viral vectors is likely to depend crucially on the individual virus and/or vector, its capacity to remain latent or persistent in brain cells, the exact nature of the infected cell type, the anatomical region infected, and the level of T cell activation. Additional characteristics, such as sex and age are also likely to be crucial determinants of the outcome of T cell mediated clearing of viral infections.

Teleologically, non-cytolytic clearing of non-dividing infected brain cells may be preferable to killing of postmitotic neurons, the alternative needs to be thoroughly examined. Direct contact or even proximity of Class I expressing cells and $CD8^+$ T cells *in situ* has only been demonstrated in isolated reports. Similarly, the notion of curative rather than cytolytic virus clearance should be regarded critically, as demonstration of apoptotic or dead CNS resident cells *in situ*, especially if the numbers are sparse are technically challenging.

The technical challenge of determining cell death *in vivo* is substantial and crucial for our understanding of the consequences for gene therapy of immune-mediated responses to vector-transduced brain cells. Immune-mediated killing cell assays rely on removing T cells from the target organ and exposing them to artificial target cells *in vitro*. While such studies have demonstrated the capacity of T cells isolated from infected brains to kill pre-selected target cells, this comes short of demonstrating that these cells indeed kill brain cells *in vivo*. *In vivo* killing assays using CFSE labeled target cells have been developed, yet their application to

CNS tissue has not yet been achieved. While such assays come closer to demonstrating that T cells can kill within the animal, whether T cells can directly kill brain target cells *in situ*, remains to be explored further. It is likely that novel methods will need to be developed to examine directly the capacity of T cells to do so.

Finally, it is likely that a combination of novel micro-anatomical imaging techniques will encourage new analyses of T cell activity in the CNS. We believe that a novel examination of the interactions of T cells with target brain cells at the individual cellular level *in vivo*, will allow new perspectives on T cell function in the CNS. New morpho-functional approaches, such as the study of immunological synaptic function *in vivo* utilizing confocal microscopy, or direct *in vivo* analysis of T cell – CNS cell interactions using two photon microscopical approaches will usher in further understandings of T cell function in the brain *in vivo*, either during the beneficial clearing of viral infections, immunopathology in response to brain infection, or autoimmune attack.

For the last ten years we and other groups have shown that immune responses against adenoviral vectors can be deleterious for brain structure in function, eventually leading to the loss of transgene expression and brain cell death. The evidence suggests that the T cell response that can identify infected cells in the brain and either eliminate them physically or functionally. Further evidence has accumulated in at least two species that T cells can actually physically eliminate vector-transduced brain cells. Should these data be correct, the logical conclusions would be to avoid the use of such vectors in clinical gene therapy trials. However, questions remain, since it is difficult to compare immune responses across species, especially, when the overall magnitude of the immune response may be what determines the clinical outcome. Further complications arise from trying to gauge the strength of the immune response in humans, who may have been exposed to wild type adenovirus decades before being exposed to gene therapy. While the threat of a deleterious immune response remains, it is almost impossible to model such responses in experimental animal species towards establishing credible expectations when translating these experiments into humans.

Two options remain. Either experiments are performed in humans never exposed to adenovirus before, or, in whom such a response cannot be detected, or novel vectors are developed specifically for clinical trials use. The first option will retain the threat of an immune response that, at a minimum, may eliminate therapeutic transgene expression, and, at a maximum compromise normal brain tissue, thereby worsening the underlying disease. The second option, is more complicated, but a number of novel viral vectors exist with a much more favorable immune profile. Within the area of adenoviral vectors, the vector structure of the HC-Adv is such that, following established gene transfer, no antigenic viral epitopes remain within infected cells; barred the development of an immune response against the therapeutic transgene, these vectors are effectively invisible to the immune system. Thus, HC-Ad are likely to represent a major step towards the implementation of gene therapy for neurological diseases, especially those therapeutic approaches requiring long term, stable, sustained expression of one or more therapeutic transgenes.

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