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ROLE OF CYTOTOXIC T LYMPHOCYTE IN ADENOVIRUS INFECTION

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Abstract: The successful development of adenovirus vectors for vaccines and gene therapy will require a better understanding of the host immune response. Using the ELISPOT assay to measure IFN-g-secreting CD8þ cells, we identify immunodominant epitopes of the adenovirus hexon and DNA-binding protein in BALB/c and C57BL/6 mice. The T-cell response to the intramuscular administration of adenovirus serotype 5 peaks within a few weeks and gradually declines but is still detectable after 12 weeks. A second administration did not substantially increase the number of reactive T cells. The CD8þ T-cell response was also similar between wild type and E1-deleted adenovirus. When B-cell-deficient mice were injected with adenovirus encoding the gene for secreted alkaline phosphatase, sera phosphatase activity was reduced more quickly in mice pre-exposed to adenovirus. These results add to the evidence that cell-mediated immunity is a substantial barrier to therapeutic adenoviral vectors and provide more quantitative tools to measure cellular immune responses to adenovirus.

Keywords: Adenovirus vectors , ELISPOT assay , T-cell response , BALB/c mice.

INTRODUCION

Human adenoviruses (HAdVs) are non-enveloped, double-stranded DNA viruses that measure 70–100 nm in diameter and have a characteristic icosahedral capsid (Lynch et al, 2016 , Lion et al, 2014 and Stephenson et al, 2020) .HAdVs can cause a broad range of clinical syndromes in childhood, typically involving the respiratory tract, conjunctiva, or gastrointestinal (GI) tract. (Lynch et al, 2016, Lion et al, 2014 and Stephenson et al, 2020). Severe, life-threatening manifestations such as respiratory failure (Shachor-Meyouhas et al, 2019, Fu et al, 2019, Lim et al, 2018 and Siew et al, 2020), myocarditis(Savon et al, 2005), or encephalitis (Schwartz et al, 2019 and Huang et al, 2013) though rare, can occur in otherwise healthy infants and children, with neonates being the most vulnerable (Ron chi et al, 2014). HAdVs are currently subdivided into seven species designated A through G within the Adenoviridae family (Lynch et al, 2016, Lion et al, 2014 and Stephenson et al, 2020). Historically, HAdVs were classified as serotypes using traditional serologic methods, serum neutralization, and hemagglutination inhibition assays (Lynch et al, 2016, Lion et al, 2014 and Stephenson et al, 2020).

With recent advances in molecular diagnostics and whole genome sequencing, over 100 distinct HAdV genotypes have now been identified (National Center for Biotechnology Information (NIH)/GenBank, 2020 and Seto et al, 2011). Infection with adenovirus occurs worldwide and has been associated with 3%-5% of cases of

acute lower respiratory tract infection (ALRI) in infants and children. (Guerrier et al, 2013). Although the positive detection rate of adenovirus in patients with respiratory infection is low, its fatal infections in immunocompromized patients arise considerable attention of pediatricians. (Nakazawa et al, 2006 and Ozbay Hoşnut et al, 2008)

The hexon protein, the most abundant capsid protein, is a strong stimulator in BALB/c (H-2d) mice (Molinier-Frenkel et al, 2002) and also contains a conserved human CD4+ epitope.(Olive et al, 2002).

Adenovirus (ADV) infections after allogeneic stem cell transplantation (SCT) are emerging as an important cause of morbidity and mortality (Chakrabarti et al, 2002). Although the immune response to adenoviral vectors has been studied extensively (Molinier-Frenkel et al, 2000 and Russell, 2000), Recently, we have shown that T cells specifically secreting interferon-y (IFN-y) can be isolated and expanded to functionally active T-cell lines in a clinical grade protocol (Feuchtinger et al, 2004).

Antigen-specific T cells are an essential part of the immune responses required to control viral infection. Frequencies of these T cells may extensively increase in response to an acute infection and normally decline after successful control of the virus (Callan et al, 1996 and Callan et al, 1998). Adenovirus (ADV) infections after allogeneic stem cell transplantation (SCT) are emerging as an important cause of ADV-specific T cells were documented in blood samples of solid organ transplant recipients and healthy individuals (Sester et al, 2002). Recently, we have shown that T cells specifically secreting interferon-y (IFN-y) can be isolated and expanded to functionally active T-cell lines in a clinical grade protocol (Feuchtinger et al, 2004).

These specific immune defenses employ B and T lymphocytes to help combat viral infection and develop long term immunological memory against recurring infections

Anti-Histamines

Another well-characterized category of drugs, anti-histamines, may also combat viral infections by influencing the way in which the virus enters the cells but without physically affecting the virus directly (Klemenstsson et al, 1990). Antihistamines can play a huge role in combating chronic diseases such as atopic asthma as well as viral infections through changing Thl /Th2 homeostasis by increasing the stimulation of Thl cells and the release of IL-2 and IFNy cytokines whilst inhibiting Th2 activation, which in turn reduces eosinophilic inflammation and prevents airway hypersensitivity in mice (Xu et al, 2018). Such studies highlight anti-histamines as good candidates for antiviral treatments as they have excellent safety profiles from previous characterizations whilst being affordable.

Vitamin D

Vitamin D is a fat-soluble steroid primarily known to help maintain healthy homeostasis in bone mineral density and general health with supplements administered to individuals at greater risk of osteoporosis and bone fractures (Hansdottir et al, 2008).

Several clinical studies have been conducted on vitamin D supplementation and their effect on respiratory infections, however, the results show conflicting data. Re-occurring respiratory infections in children showed a reduction in re-infections after six weeks of vitamin D supplements (Laaksi et al, 2007).

Vitamin D can also influence the adaptive immune system, particularly T lymphocyte regulation via the upregulation of Th2 cytokines associated with an anti-inflammatory response, whilst simultaneously stimulating the differentiation and expansion of regulatory T-cells through VDR activation. Mechanisms for vitamin D induced antiviral activity are well-described (Teymoori et al, 2019 and Lee et al, 2020), however, deciphering these diverse biological activities in the context of different viral infections requires further investigation including validated markers of immune modulation (Ramamoorthy et al, 2016).

Dexamethasone

Dexamethasone is a corticosteroid affecting the hypothalamic-pituitary-adrenal axis (HPA) for the regulation of metabolism, development, homeostasis, and cognition (Coutinho et al, 2011). It targets inflammation by binding to the glucocorticoid receptor (GR) on the cell membrane, influencing translocation, and promoting immunosuppression by preventing the extension of the cytokine storm (De et al, 2002). This provides a rapid relief of inflammation and hence its use extends to the treatment of rheumatoid arthritis (Nair et al, 2018), Thus, dexamethasone can be used to prevent the persistence and maintenance of the immune system (Giles et al, 2018). Therefore, there are alterations in the Th ratio that must be due to persistence of the immune response.

The Th bias can be seen to influence disease severity.

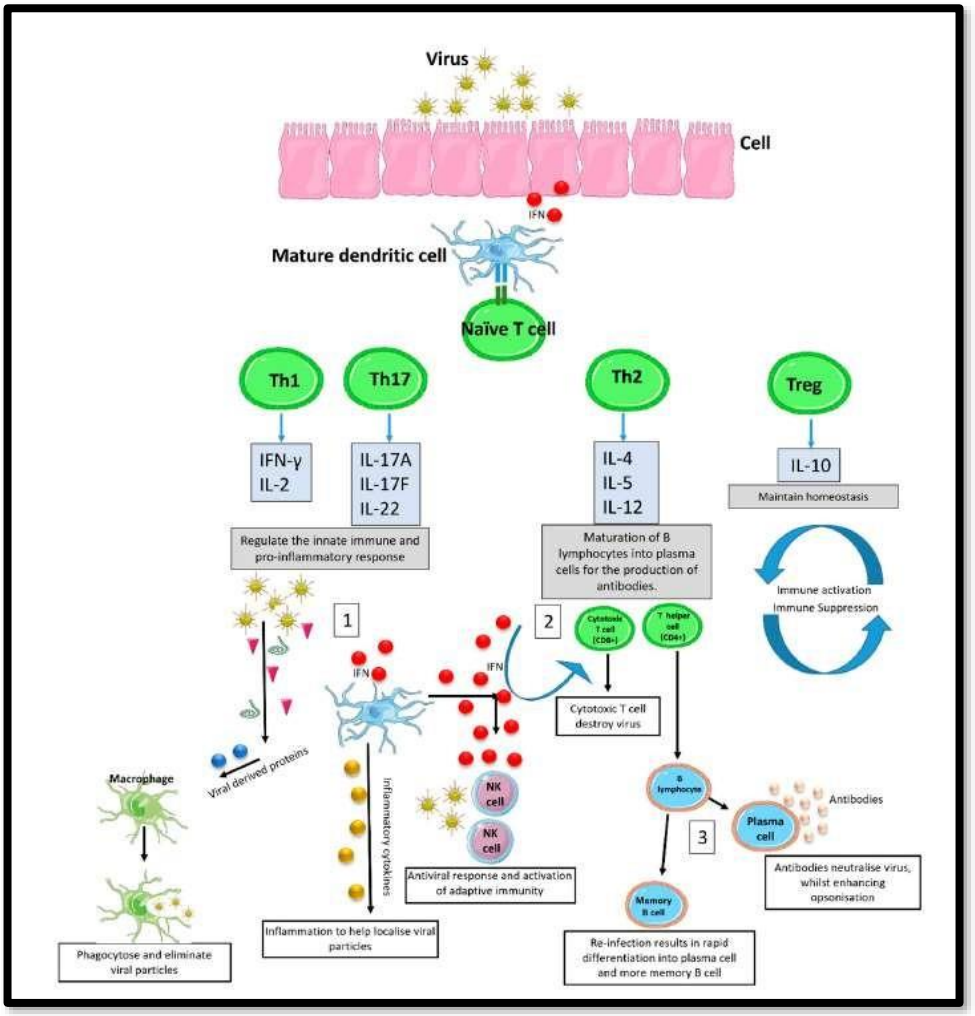

Figure 1. Immune response to viral infection. Viral infections elicit an immune response by first activating the innate immune system. Infected cells release IFN and pro-inflammatory cytokines that activate natural killer cells to destroy the viral infection.)**Kaiko *et al*, 2008(.**

2. Causes of Deviant Immune Response in Viral Infections

Much focus has been given to the identification of specific human gene variants responsible for enhanced susceptibility or resistance to viral infection and it would be remiss of us not to include the genetic underpinnings that control viral infection outcomes; however, these have been reviewed elsewhere (Kenney et al., 2017). Briefly, the comparison of infected versus uninfected individuals have elucidated specific genetic factors responsible for divergent immune responses to specific viruses resulting in variability in both an individual's susceptibility and outcome (examples in Table 1). (Singh et al, 2007 and DeWit et al, 2016).

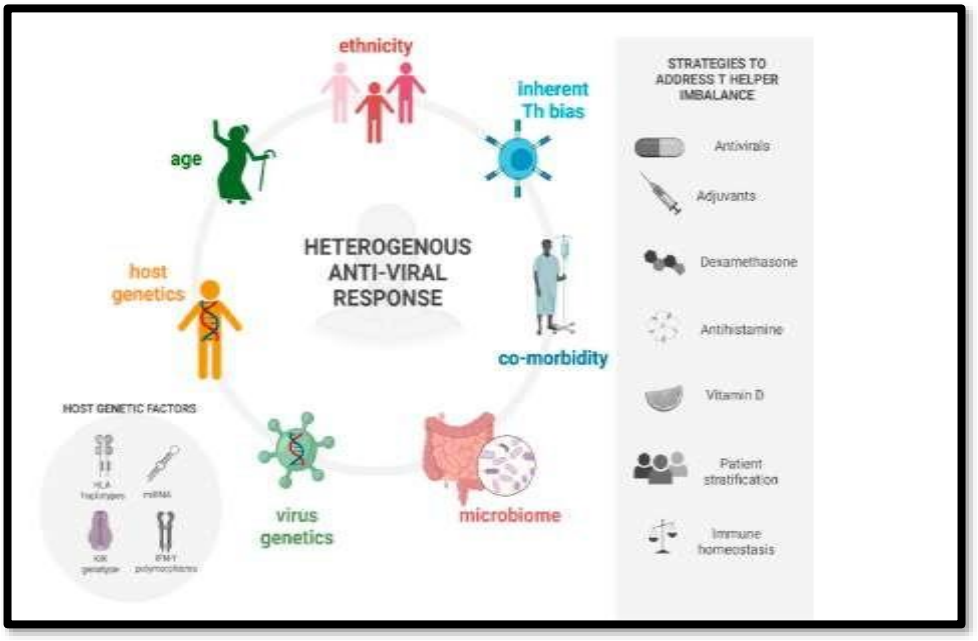

Figure2. Aschematic summarizing the causes of a hetrogenous anti-viral immune response and possible strategies to address T helper cell imbalance. Created with biorender. **(Kenney *et al.*, 2017).**

THE DESIGN AND METHOD

Animals

Female BALB/c, C57BL/6, Jh and SCID mice were obtained from Taconic Laboratories at approximately 4–6 weeks old and given food and water ad lib. For i.m. vaccinations, the mice were injected with 50 ml into each rear quadriceps muscle. All experiments were approved by the Institutional Animal Care and Use Committee.

Cell lines and viruses

Cell culture media and reagents were obtained from Invitrogen Corp. unless stated otherwise. WtAd5 was obtained from the ATCC and propagated in Hela cells. The Hela cells were grown in monolayer in minimum essential medium, alpha medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 4 mM L-glutamine and 10% (v/v) UV-irradiated fetal bovine serum. AdE1- and Ad5SEAP were propagated in PER.C6 cells (Fallaux et al, 1998) grown in monolayer in William's Medium E Modified (Hyclone) supplemented as above. AdE1- and Ad5SEAP are replication-incompetent first generation vectors containing E1 region deletions from nt 342 to 3523 and in the case of Ad5SEAP an E3 deletion from nt 28 133 to 30 818. In Ad5SEAP, the secreted alkaline phosphatase (SEAP) transgene (Berger et al, 1988) is located in the E1 region in the E1 antiparallel orientation. Transcription of Adenovirus T-cell response T McKelvey et al the transgene is driven by the human cytomegalovirus promoter including Intron A (Chapman et al, 1991) and terminated using the bovine growth hormone polyadenylation signal.

Peptides

Were custom synthesized by Research Genetics.

ELISPOT

The ELISPOT assay was performed as previously descibed. (Klinman et al, 1994) Spleens were harvested from BALB/c, Jh or C57BL/6 mice, minced in K media (RPMI medium 1640 supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 4 mM L-glutamine, 55 mM b-mercaptoetha- nol, 10 mM HEPES and 10% fetal bovine serum) over a mesh insert and the RBCs lysed with AKC lysing buffer. The cells were diluted to 107 cells/ml in K media and 100 ml of the cell suspension was added to 100 ml of the appropriate antigen at 2 mg/ml in the well of a 96-well microplate with a nitrocellulose bottom coated with capture antibody (purified rat anti-mouse IFN-g, Phar- Mingen). The plates were incubated for 18–22 h at 371C in 5% CO2, washed and 100 ml/well biotinylated rat anti- mouse IFN-g (PharMingen) was added. The plates were washed again and 100 ml/well streptavidin-AP conjugate (PharMingen) was added. Color was developed by adding 100 ml/well 1-STEP NBT-BCIP (Pierce) for 5–10 min. Spots were counted and the spot-forming cells (SFC) per 106 cells were calculated. The nonspecific mitogen concanavalin A was the positive control and produced a solid color in all assays.

SEAP assay

Blood was collected from mice via the tail vein and sera collected after centrifugation. The sera was heat-treated for 30 min at 65 C to inactivate endogenous alkaline phosphatase activity and the secreted alkaline phospha-

tase activity measured using a Tropix Phospha-Light luminescence assay (Applied Biosystems). The light output over 5s was measured on a Dynex MLX luminometer and relative luminescence output con- verted to ng/ml of SEAP by linear regression of an alkaline phosphatase (Sigma) standard curve.

RESULT

Identification of adenovirus epitopes The CD8 þ epitopes of adenovirus type 5 hexon and DNA-binding protein were identified as shown in Figure 1. The primary sequence of the hexon protein was divided into 24-mer peptides overlapping by 12 amino acids, which were then grouped into pools of 10 peptides and tested in the ELISPOT assay against mice vaccinated with wild-type Ad5 (wtAd5). The DNA-binding protein was divided into 22-mer peptides overlapping by 11 amino acids, which were also grouped into pools of 10 peptides and tested in the ELISPOT assay. Individual peptides from pools that scored positive were then tested in the assay against CD4 þ - and CD8 þ -depleted splenocytes. In BALB/c mice, the hex21 24-mer peptide (amino acids 481–504, LPDKLKYSPSNVKISDNPNTYDYM) and the dbp43 22-mer peptide (amino acids 409–430, LGRQLPKLTPFALSNAEDLDAD) were identified to contain strong CD8 þ epitopes. In C57BL/6 mice, the dbp43 22-mer peptide (amino acids 409–430, LGRQLPKLTPFALSNAEDLDAD) was identified to have the principal CD8 þ epitope. Shorter 9-mer peptides (hex3: KYSPSNVKI, dbp7: LPKLTPFAL, dbp43: FALSNAEDL) were synthesized and tested for each of the three identified epitopes. Each of these peptides gave signals as high or higher than the longer peptides and was used for all subsequent assays.

BALB/c mice were vaccinated with 108 viral particles of wtAd5 and 4 weeks later tested in the ELISPOT assay against the hex and dbp peptides. The results of 10 vaccinated mice and three unvaccinated mice tested are shown in Table 1. In vaccinated mice, the SFC/106 ranged from 151 to 570 with an average of 310 and a s.d. of 130 for the hex3 peptide. Against the dbp7 peptide, the number of spot-forming cells per million ranged from 321 to 628 with an average of 456 and a s.d. of 111. Given the range of responses, we found it appropriate to conduct most assays using cells from three pooled spleens.

Immune response of wild type versus E1- deleted adenovirus

Most of our studies were performed with wild-type Ad, whereas most of the gene therapy and vaccine studies are conducted using replication defective, E1 region adenovirus vectors. We compared wild type and E1 region virus in the ELISPOT assay in order to examine the effect of the deletion of the E1 region on CMI. BALB/c and C57BL/6 mice were primed with 108 viral particles of wtAd5 or Ad5E1, boosted 4 weeks later with the same vector, and tested t3 or 6 weeks later in ELISPOT assays. The results are displayed in Table 2. For both strains, deletion of the E1 region did not significantly change the T-cell responses. Functional role of T cells in B-cell-deficient mice and pre-existing immunity we also investigated the functional role of T cells in the antiadeno immune response using adenovirus vectors encoding the secreted alkaline phosphatase (SEAP) gene.

DISCUSSION

We demonstrate that BALB/c and C57BL/6 mice have strong cell-mediated immune responses to adenovirus serotype 5 proteins as determined with the ELISPOT IFN-g assay. BALB/c mice (H-2d) recognize CD8 þ epitopes in the adenovirus hexon and DNA-binding protein. C57BL/6 mice (H-2b) mount a strong CD8 þ response against an epitope in the adenovirus DNA binding protein. Possible reasons for the differences seen in the response to the DNA-binding protein include the source of antigen, and the nature of the assay. Our source of antigen was peptides, which may be more sensitive than the vaccinia virus encoding the entire DNA-binding protein as used in the previous study. Also, we used the ELISPOT assay that may be more sensitive than the CTL assays performed previously. In people, an immunodominant hexon CD4 þ epitope has been previously defined in HLA-A2 donors, (Olive et al, 2002)and adenovirus capsid proteins have previously been shown to be targets for cytotoxic T lymphocytes.(Molinier-Frenkel et al, 2000) We further characterized the magnitude and time course of the T-cell response in this model as measured in the ELISPOT assay. A dose-dependent increase in the number of IFN-g cells was seen over a range of 105 –108 viral particles. Higher doses did not induce a stronger ELISPOT response to the target peptides (data not shown). Also, the data did not show a consistent increase in the number of CD8 þ T cells secreting interferon gamma by a prime/boost regimen over a single vaccination. The failure of repeated injections of adenovirus to boost the signal in the ELISPOT assay is surprising given that we have seen increases in ELISPOT signals to transgenes after repeated vaccinations in similar mouse experiments.

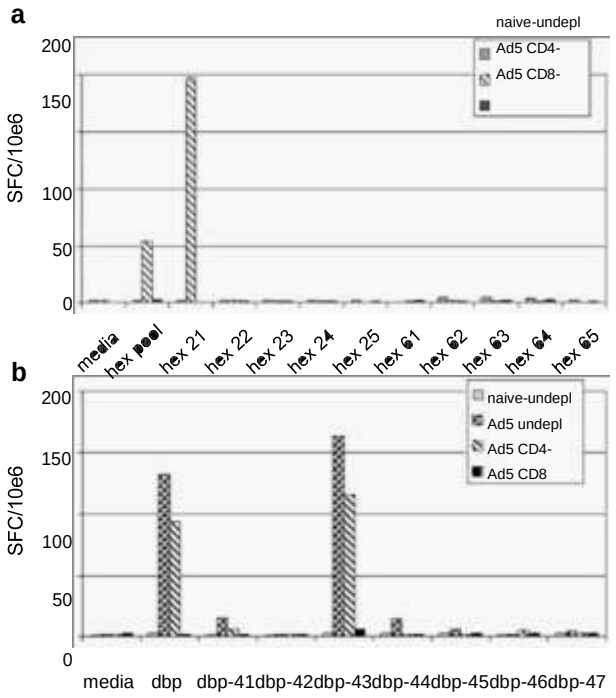

Figure 1 Identification of mouse CD8 þ adenovirus protein epitopes. Mice were vaccinated with 108 viral particles of wtAd5, then boosted 4 weeks later with the same dose. After 4 weeks, the spleens were harvested and the splenocytes were tested in an ELISPOT assay. The number of SFC per 106 cells (SFC/106) tested for the positive peptides and the peptide pools in which they were screened are shown. (a) The hexon protein in BALB/c mice (DNA-binding protein not shown). (b) The DNA-binding protein in C57BL/6 mice.

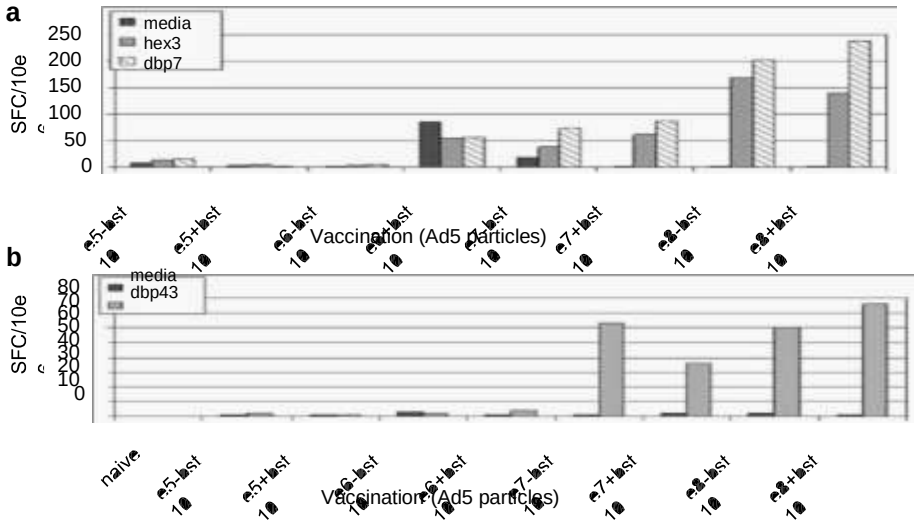

Figure two Dose response of mice to adenovirus vaccination. Mice were injected with 105 –108 viral particles of wtAd5. After 4 weeks, half were boosted with the same dose. After an additional 4 weeks, the cells from three spleens were pooled and the number of responding cells was determined in the ELISPOT assay. (a) BALB/c response to the hex3 and dbp7 peptides. (b) C57BL/6 response to the dbp43 peptide.

Table 2 Immune response of wild-type versus E1 adenovirus

Antigen	Naive	wtAd5	Ad5E1-
BALB/c – 3 weeks postboost			
Media	10	13	18
hex3	17	132	60
dbp7	21	158	128

C57BL/6 – 6 weeks postboost			
Media	47	18	2
dbp43	14	380	329

BALB/c and C57BL/6 mice were primed with 108 viral particles of wild type or Ad5E1 and boosted 4 weeks later with the same. After 3 or 6 weeks, the cells from three spleens were pooled and tested in the ELISPOT assay against hex or dbp peptides. Data from the BALB/c mice is from 6 weeks post boost and is 3 weeks post boost for C57BL/6 mice.

CONCLUSION

Blood was collected from mice via the tail vein and sera collected after centrifugation. The sera was heat-treated for 30 min at 651C to inactivate endogenous alkaline phosphatase activity and the secreted alkaline phosphatase activity measured using a Tropix Phospha-Light luminescence assay (Applied Biosystems). The light output over 5 s was measured on a Dynex MLX luminometer and relative luminescence output converted to ng/ml of SEAP by linear regression of an alkaline phosphatase (Sigma) standard curve.

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