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Chronic inhibition of cyclooxygenase-2 attenuates antibody responses against vaccinia infection

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ABSTRACT

Generation of optimal humoral immunity to vaccination is essential to protect against devastating infectious agents such as the variola virus that causes smallpox. Vaccinia virus (VV), employed as a vaccine against smallpox, provides an important model of infection. Herein, we evaluated the importance cyclooxygenase-2 (Cox-2) in immunity to VV using Cox-2 deficient mice and Cox-2 selective inhibitory drugs. The effects of Cox-2 inhibition on antibody responses to live viruses such as vaccinia have not been previously described. Here, we used VV infection in Cox-2 deficient mice and in mice chronically treated with Cox-2 selective inhibitors and show that the frequency of VV-specific B cells was reduced, as well as the production of neutralizing IgG. VV titers were approximately 70 times higher in mice treated with a Cox-2 selective inhibitor. Interestingly, Cox-2 inhibition also reduced the frequency of IFN-γ producing $CD4^+$ T helper cells, important for class switching. The significance of these results is that the chronic use of non-steroidal anti-inflammatory drugs (NSAIDs), and other drugs that inhibit Cox-2 activity or expression, blunt the ability of B cells to produce anti-viral antibodies, thereby making vaccines less effective and possibly increasing susceptibility to viral infection. These new findings support an essential role for Cox-2 in regulating humoral immunity.

1. Introduction

Antibodies are essential mediators of anti-viral immunity, and important for successful vaccination. Effective vaccines are in demand worldwide, as many fail to strongly stimulate B cell production of protective antibodies [1,2]. The smallpox vaccine, consisting of live vaccinia virus (VV), is an example of a functional vaccine that elicits a potent immune response, resulting in long lasting B cell memory that can last upwards of 75 years [3–5]. Due to the eradication of smallpox worldwide in 1980, the smallpox vaccine is no longer administered to the general public [6]. However, it is still administered to military personnel, as well as to some health care workers. The threat of bioterrorism has peaked interest in the study of pathogens, such as variola, the causative agent of smallpox, which could be weaponized. It is, therefore, important to understand vaccine-induced immune responses, so that those that are weakly immunogenic, can be improved, and factors that diminish immunity may be avoided.

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to alleviate the side-effects (pain, fever, swelling, edema, *etc.*) of vaccination and to reduce fever and pain associated with infections. NSAIDs function by inhibiting cyclooxygenases-1 and -2. Cyclooxygenases (Cox) are necessary for the production of prostaglandins, which regulate inflammation [7]. More recently, Cox-2 selective small molecule inhibitors have been developed as treatments for rheumatoid arthritis and for chronic pain. Cox-2 is highly elevated during inflammation and plays an important role in immune regulation [8–10]. B lymphocytes express Cox-2 after activation with stimuli such as CD40 ligand, CpG oligodeoxynucleotides and BCR engagement [11,12]. Cox-2 expression and activity was also shown to be essential for optimal antibody production *in vitro* [11,12]. Cox-2 deficient mice exhibited impaired B cell responses following vaccination with non-infectious human papillomavirus-16 virus-like particles (HPV-16 VLP) [13]. However, whether Cox-2 plays a vital role in the

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humoral immune response to *live* virus infection is currently unknown.

Protection against viruses requires both the humoral and cellular arms of the immune system. Antibodies are necessary for viral clearance and prevention of viral replication. Monkeys given anti-CD20 treatment to deplete B cells, died after challenge with monkeypox, but were spared if passive antibody transfer was performed [14,15]. Xu et al. provide evidence that both B cell deficient mice and mice depleted of $CD4^+$ T cells had highly impaired VV clearance, both due to a lack of antibody production [16]. In the absence of $CD4^+$ T cell help, B cells fail to undergo class switching and somatic hypermutation. These processes are important for generation of highly specific neutralizing antibodies.

The purpose of the present study was to determine, using Cox-2 knockout mice and mice treated with Cox-2 selective inhibitors, whether antibody production would be adversely affected in response to live VV infection. Further, we hypothesized that $CD4^+$ T cell responses, critical for B cell class switching and production of neutralizing antibodies, to VV would also be impaired. Our new results support the concept that chronic use of Cox-2 selective inhibitors during live virus infection will attenuate humoral immunity, possibly making patients more susceptible to infectious agents such as variola.

2. Materials and methods

2.1. Virus

The Western Reserve strain of vaccinia virus was grown in 143B fibroblasts.

2.2. Cox-2 selective inhibitors

SC-58125 (Cayman Chemical), a celecoxib analogue, and NS-398 (Cayman Chemical, Ann Arbor, MI) were dissolved in DMSO and diluted to 10% in an aqueous solution of hydroxypropyl methyl cellulose (HPMC). Two hundred microliters of the HPMC/Cox-2 inhibitor solution were given to mice via oral gavage two times per week. SC-58125 was administered at 5 mg/kg and NS-398 at 10 mg/kg. DMSO/HPMC was used as the vehicle control.

2.3. Mice and infection protocols

Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME), Cox-2 deficient mice (B6.129P2-Ptgs2^{tm1Unc}) and wild-type controls were purchased from Taconic Farms (Hudson, NY). Congenic B6-Ly5.2/Cr mice (NCI, Frederick, MD) were used for antigen presenting cells (APCs) in intracellular cytokine staining (ICS) and IFN-γ ELISPOT assays. Approval of all protocols was obtained from the University of Rochester animal care and use committee. C57BL/6 mice were used in three different infection protocols. All mice were infected i.p. with 1×10^6 PFU of the Western Reserve strain of VV. Cox-2 deficient mice and wild-type controls were infected on day 0 and sacrificed on day 28. C57BL/6 mice were infected on day 0 and were chronically treated with SC-58125 starting 6 days prior to infection and ending on day 27. C57BL/6 mice were infected with VV on day 0 and were acutely treated with vehicle, NS-398 or SC-58125 starting on day 0 and ending on day 7. Plasma, bone marrow cells and splenocytes were harvested from infected mice at various time points. Four mice were used per treatment in each experiment.

2.4. Virus inactivation

Inactivation of VV was performed as previously described [5]. VV stocks (4×10^8 PFU/mL) were incubated with 4′-aminomethyltrioxsalen (10 μg/mL) (Calbiochem, La Jolla, CA) for 10 min at room temperature (RT). VV stocks were then placed in 6-well plates and UV inactivated in a Stratagene 2400 UV Crosslinker (Stratagene, La Jolla, CA) at a setting of 3.0 J/cm^2. Inactivation was confirmed with plaque assays. Inactivated VV stocks were stored at −80 °C.

2.5. VV-specific ELISAs

Ninety six-well ELISA plates were coated and incubated overnight with inactivated VV diluted 1:100 in 1× PBS, 0.1% BSA. Plates were blocked with 1× PBS, 1.0% BSA for 1 h. Serial dilutions of mouse plasma were diluted in 1× PBS, 0.1% BSA and incubated for 2 h at RT. Following washing, plates were incubated for 1 h with alkaline phosphatase-conjugated IgM, IgG, IgG1, IgG2a, IgG2b or IgG3 secondary antibodies (1:2000) (Southern Biotech, Birmingham, AL). ELISAs were developed with the *p*-nitrophenyl phosphatase substrate kit (Pierce/Thermo Fisher Scientific, Rockford, IL) and O.D. 405 nm values were determined on a microplate reader (Bio-Rad, Hercules, CA).

2.6. VV-specific ELISPOTs

Ninety six-well ELISPOT plates (Millipore, Billerica, MA) were coated and incubated overnight with inactivated VV diluted 1:20 in 1× PBS, 2.0% BSA, 0.1% Tween-20. Plates were blocked with RPMI 1640 (GIBCO/Invitrogen) supplemented with 5% FBS, 2 mM L-glutamine, 5×10^5 M 2-ME, 10 mM HEPES and 50 μg/mL gentamicin at 37 °C for 1 h prior to addition of cells. Splenocytes and bone marrow harvested from mice were processed into single cell suspensions and incubated in ELISPOT plates for 6 h (37 °C, 5% CO_2). Following this incubation, plates were washed and incubated overnight with alkaline phosphatase-conjugated IgM, IgG, IgG1, IgG2a, IgG2b or IgG3 secondary antibodies (1:1000) (Southern Biotech). ELISPOTs were developed with Vector substrate kit (Vector Labs, Burlingame, CA) and counted on a CTL plate reader, using ImmunoSpot software (Cellular Technologies Ltd., Shaker Heights, OH). Protocols were modified based on published protocol by Crotty et al. [17].

2.7. Plaque reduction assays

143B fibroblasts (6×10^5/well) cultured in MEM (GIBCO/Invitrogen) supplemented with 8% FBS, 2 mM L-glutamine, 5×10^5 M 2-ME, 10 mM HEPES and 50 μg/mL were plated in 6-well plates and allowed to grow to confluency at 37 °C overnight. Western Reserve strain VV was diluted 1×10^{-6} (400 PFU/mL) in MEM, 2.5% FBS. Five hundred microliters of this prep was incubated with dilutions of mouse plasma for 2 h to allow for neutralization to occur. Cells were then incubated with 500 μL of VV/plasma for 1.5 h. After this incubation, 1.5 mL of MEM, 2.5% FBS was added to cultures and cells were left at 37 °C for 48 h. Media was removed and cells were stained with a 20% EtOH, 0.1% crystal violet solution. Plaques were counted and are represented as a percent of plaques in the absence of plasma.

2.8. Antibody production assays

Splenocytes (1×10^6) were isolated from naive C57BL/6 mice and cultured without stimuli for 48 h. Cells were treated with DMSO vehicle, SC-58125 or NS-398 at various concentrations.