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IL-21R expression on CD8+ T cells promotes CD8+ T cell activation in coxsackievirus B3 induced myocarditis

Wei Liu [a], Oliver Dienz [b], Brian Roberts [b], Mohamad Moussawi [b], Mercedes Rincon [b], Sally A. Huber [b,*]

[a] The First Affiliated Hospital of Harbin Medical University, 23 Youzheng Street, Harbin, Heilongjiang Province, 150001, China
[b] University of Vermont, 208 South Park Drive, Colchester, VT 05446, USA

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ABSTRACT

IL-21 is a multi-functional cytokine which can promote survival, proliferation and activation of T and B lymphocytes including CD8 T cells. Previous studies have shown that autoimmune CD8+ T cells are the primary pathogenic effector cell in coxsackievirus B3 (CVB3) induced myocarditis in C57Bl/6 mice. To evaluate the role of IL-21 in promoting CD8+ T cell mediated cardiac injury in myocarditis, C57Bl/6 and IL-21RKO mice were infected with CVB3. IL-21RKO mice developed significantly less myocarditis than C57Bl/6 animals although cardiac virus titers were equivalent between the mouse strains. Numbers of CD8+IFNγ+ cells were decreased in IL-21RKO mice but numbers of either CD4+IFNγ+ or CD4+IL-4+ cells were not significantly different from C57Bl/6 animals indicating a selective effect of IL-21 signaling on the CD8+ T cell response. To confirm that IL-21 signaling exclusively functions at the level of the CD8+ T cell in CVB3 induced myocarditis, purified CD8+ cells were isolated from either C57Bl/6 or IL-21RKO donors and adoptively transferred into CD8KO recipients prior to CVB3 infection. CD8KO recipients given either C57Bl/6 or IL-21RKO CD8+ cells showed equivalent reconstitution of the CD8+ cells in the spleen but the recipients given C57Bl/6 CD8+ cells showed significantly greater myocarditis than recipients of IL-21RKO CD8+ cells. These data demonstrate that IL-21 signaling directly in the CD8+ cell population is required for CVB3-induced myocarditis.

Introduction

Myocarditis is an inflammation of the cardiac muscle which follows microbial infections (Huber, 2008). Among viruses, enteroviruses including coxsackie B viruses are common etiologic agents (Bowles et al., 2002, 2003). Although infectious agents act as a trigger for myocarditis, there is considerable debate as to the actual mechanism(s) of myocardial injury. Viruses directly cause cellular dysfunction either through induced cell death, shut down of cell RNA and protein synthesis or viral protease cleavage of contractile proteins (Badorff et al., 1999; Rueckert, 1996). Additionally, cytokines such as IL-1β, IL-6 and TNFα which are elicited from resident cells in the heart subsequent to infection can suppress contractility leading to cardiac dysfunction (Freeman et al., 1998). Finally, host immune responses to infection may kill myocytes leading to cardiac stress. Host response can be specifically directed toward virally infected cardiocytes or infection can trigger autoimmunity to cardiac antigens (autoimmunity) which destroys both infected and uninfected myocytes (Rose, 2008). Autoantibodies and cytolytic T cells to heart antigens are found in patients with myocarditis (Maisch, 1989; Maisch et al., 1993), and myocardial inflammation has been shown by adoptive transfer of anti-myosin antibodies in mice (Liao et al., 1993, 1995). Immunization of mice with cardiac myosin results in myocarditis which closely resembles the disease resulting from coxsackievirus B3 (CVB3) infection (Fairweather et al., 2001, 2005; Kaya et al., 2002). T cells are primarily responsible for cardiac injury in the CVB3 model of myocarditis in mice (Woodruff and Woodruff, 1974). Both CD4+ and CD8+ T cells are activated during infection (Lodge et al., 1987) but respond to different types of antigen. Evidence indicates that the CD4+ cell response recognizes infected but not uninfected myocytes while the CD8+ effector cells react only to uninfected myocytes through recognition of cardiac myosin (Guthrie et al., 1984; Huber and Cunningham, 1996; Huber and Gauntt, 2000; Huber and Lodge, 1984, 1986; Huber et al., 1987). Of the two T cell subsets, the CD8+ cells are the primary mediator of cardiac injury. However, without CD4+ cell activation, the autoimmune CD8+ cell response is prevented (Huber et al., 2002).

A major question is what environmental factors during infection lead to autoimmune CD8+ T cell activation. IL-21 is a multi-functional cytokines which is a member of the type 1 cytokine family (Parrish-Novak et al., 2000) and is primarily produced by CD4+ and natural killer T cells (Coquet et al., 2007; Nurieva et al., 2007). IL-21 signals through

* Corresponding author. Fax: +1 802 656 8968.
E-mail addresses: doctor_Liuwei@yahoo.com.cn (W. Liu), oliver.dienz@uvm.edu (O. Dienz), brian.roberts@uvm.edu (B. Roberts), Mohamad.moussawi@uvm.edu (M. Moussawi), Mercedes.rincon@uvm.edu (M. Rincon), sally.huber@uvm.edu (S.A. Huber).

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the IL-21R which consists of a specific IL-21Rα chain and the common γ chain used by multiple other cytokines (IL-2, IL-4, IL-7, IL-9 and IL-15) (Spolski and Leonard, 2008). IL-21 signaling primarily activates the Jak/STAT pathway with STAT1 and STAT3 being the predominant targets (de Totero et al., 2008). It was initially proposed that IL-21 was also a Th17-derived cytokine and was required for Th17 differentiation and development of EAE (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). However, recent studies performing intracellular staining for cytokines showed that the IL-21-producing cells in Th17 cells represent an independent subset from the IL-17-producing cells, and that Th17 cell differentiation and EAE are independent of IL-21 (Coquet et al., 2008; Sonderegger et al., 2008; Suto et al., 2008). Some studies indicate that IL-21 can cooperate with IL-27 in activation of regulatory type 1 (Tr1) cells (Pot et al., 2010) and also suppress immune responses through promotion of IL-10 expression (Spolski and Leonard, 2011; Spolski et al., 2009). Thus, this cytokine has both pro- and anti-inflammatory functions depending upon cytokine environment. IL-21 is the primary cytokine secreted by CD4 T follicular helper (Tfh) cells, a newly characterized subset of effector CD4 T cells that localize in the germinal centers (GC), and are essential for germinal center formation through a direct effect on B cells (Bryant et al., 2007; Chtanova et al., 2004; Fazilleau et al., 2009; McHeyzer-Williams et al., 2009). Recent studies have shown that IL-21 is not required for the generation of Tfh cells, but Tfh cell-derived IL-21 is essential for formation of GC by acting directly on B cells (Linterman et al., 2010; Zotos et al., 2010). Another major function of IL-21 is on CD8+ cell activation, proliferation, survival and cytolytic activity (Frederiksen et al., 2008; Li et al., 2005; Novy et al., 2011; Zeng et al., 2005). Also, IL-21 has been shown to promote conversion of CD8+ effector to memory cells (Barker et al., 2007, 2010; Cui et al., 2011).

Based on the abundant literature that IL-21 promotes autoimmune and CD8+ T cell responses, the current study investigated whether IL-21 is essential to induction of autoimmune CD8+ T cells in the CVB3 model of myocarditis. The studies demonstrate that mice lacking the IL-21R fail to develop myocarditis despite high virus titers. CD8+ cell responses are not exclusively affected by IL-21R deficiency. While total numbers of CD4+ cells are increased in the spleens of infected and uninfected IL-21RKO mice, CD4+IFNγ+ cell responses are reduced in the absence of IL-21 signaling. CD8+ T cell activation is substantially reduced, but since CD8+ cell responses during CVB3 infection depend upon IFNγ produced by CD4Th1 cells, this effect could be due either to direct defects in IL-21 signaling in the CD8+ cell population or to indirect effects of IL-21 defective signaling in the CD4+ cells. Adoptive transfer of CD8+ IL-21RKO cells into CD8KO recipients demonstrates that IL-21 signaling directly through the CD8+ effector population promotes myocarditis susceptibility.

Materials and methods

Mice

Male C57Bl/6 and C57Bl/6 CD8KO (B6.129S2-*Cd8a*^{tm1Mak}/J) mice were purchased from Jackson Laboratories, Bar Harbor ME. IL-21RKO transgenic mice on the C57Bl/6 background were previously described (Ozaki et al., 2002) and a breeding colony of these mice was maintained at the University of Vermont. All mice were 5–7 weeks of age when infected. All of the studies have been reviewed and approved by the University of Vermont Institutional Animal Care and Use Committee.

Virus

The H3 variant of CVB3 was made from an infectious cDNA clone as described previously (Knowlton et al., 1996).

Infection of mice

Mice were injected intraperitoneally (i.p.) with 10^2 plaque forming units (PFU) virus in 0.5 ml PBS. Animals were killed when moribund or 7 days after infection.

Virus titers

Hearts were asceptically removed from the animals, weighed, and homogenized in RPMI 1640 medium containing 5% fetal bovine serum (FBS), L-glutamine, streptomycin and penicillin. Cellular debris was removed by centrifugation at 300 ×*g* for 10 min. Supernatants were diluted serially using 10-fold dilutions and titered on Hela cell monolayers by the plaque forming assay (Van Houten et al., 1991).

Histology

Tissue was fixed in 10% buffered formalin for 48 h, paraffin embedded, sectioned and stained by hematoxylin and eosin. Image analysis of cardiac inflammation was done blindly on coded slides to determine the percent of the myocardium inflamed (Knowlton et al., 1996). Two sections were analyzed per heart and showed less than 10% variation between the sections in percent inflammation. If greater than 10% variation was observed, then an additional 2–5 sections per heart were analyzed until the SEM from the mean of the sections was <10%. The mean percent inflammation from the multiple sections was used as the percent inflammation for the individual mouse.

Isolation of lymphocytes

Spleens were removed and pressed through fine mesh screens. Lymphoid cells were isolated by centrifugation of cell suspensions on Histopaque (Sigma). Isolation of purified CD8+ T cells was done using the BD Imag™ Mouse CD8 T Lymphocyte Enrichment kit according to manufacturer's directions (BD Biosciences, Rockville, MD).

Intracellular cytokine staining

Details for intracellular cytokine staining have been published previously (Huber et al., 2001). 10^5 spleen cells were cultured for 4 h in RPMI 1640 medium containing 10% fetal bovine serum, antibiotics, 10 μg/ml of brefeldin A (BFA; Sigma), 50 ng/ml phorbol myristate acetate (PMA; Sigma), and 500 ng/ml ionomycin (Sigma). The cells were washed in PBS–1% bovine serum albumin (BSA; Sigma) containing BFA, incubated on ice for 30 min in PBS–BSA–BFA containing a 1:100 dilution of Fc Block, Alexa647-anti-CD8a and PerCP-Cy5.5 anti-CD4 (clone GK1.5) or PerCP-Cy5.5 and Alexa647 rat IgG2b (clone A95-1, isotype controls). The cells were washed once with PBS–BSA–BFA, fixed in 2% paraformaldehyde for 10 min, then resuspended in PBS–BSA containing 0.5% saponin, Fc Block and 1:100 dilutions of the following antibodies or isotype control immunoglobulins: PE anti-IFNγ (cloneXMG1.2) or PE-rat IgG1 (clone R3-34); or FITC-anti-IL-4 (clone 11B11) or FITC-rat IgG1 (clone R3-34) and incubated for 30 min on ice. All antibodies were from BD Biosciences/Pharmingen. Cells were analyzed using a BD LSR II flow cytometer with a single excitation wavelength (488 nm) and band filters for PerCP-Cy5.5 (695/40 nm), FITC (525 nm), and PE (575 nm). The excitation wavelength for Alexa 647 is 643 nm and a band filter of 660/20 nm. The cell population was classified for cell size (forward scatter) and complexity (side scatter). At least 10,000 cells were evaluated. Positive staining was determined relative to isotype controls. To determine the number of individual cell populations, the total number of viable cells was determined by trypan blue exclusion. Following flow cytometry, the % of a subpopulation staining with a specific antibody was multiplied by the total number of spleen cells.