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Natural Killer Cells Limit Cardiac Inflammation and Fibrosis by Halting Eosinophil Infiltration

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Myocarditis is a leading cause of sudden cardiac failure in young adults. Natural killer (NK) cells, a subset of the innate lymphoid cell compartment, are protective in viral myocarditis. Herein, we demonstrated that these protective qualities extend to suppressing autoimmune inflammation. Experimental autoimmune myocarditis (EAM) was initiated in BALB/c mice by immunization with myocarditogenic peptide. During EAM, activated cardiac NK cells secreted interferon γ, perforin, and granzyme B, and expressed CD69, tumor necrosis factor–related apoptosis-inducing ligand treatment, and CD27 on their cell surfaces. The depletion of NK cells during EAM with anti-asialo GM1 antibody significantly increased myocarditis severity, and was accompanied by elevated fibrosis and a 10-fold increase in the percentage of cardiac-infiltrating eosinophils. The resultant influx of eosinophils to the heart was directly responsible for the increased disease severity in the absence of NK cells, because treatment with polyclonal antibody asialogangloside GM-1 did not augment myocarditis severity in eosinophil-deficient ΔdoubleGATA1 mice. We demonstrate that NK cells limit eosinophilic infiltration both indirectly, through altering eosinophil-related chemokine production by cardiac fibroblasts, and directly, by inducing eosinophil apoptosis *in vitro*. Altogether, we define a new pathway of eosinophilic regulation through interactions with NK cells. *(Am J Pathol 2015, 185: 847–861; http://dx.doi.org/10.1016/j.ajpath.2014.11.023)*

Myocarditis is a leading cause of sudden cardiac failure in individuals <40 years, with 9% to 16% of cases progressing to inflammatory dilated cardiomyopathy.[1–3] Necrotizing eosinophilic myocarditis, a subset of myocarditis, is characterized by extensive cardiac eosinophilic infiltration, pronounced cardiomyocyte death, and higher fatality rates.[4–9] Correlations between eosinophil frequency and poor clinical outcomes have been reported in other chronic inflammatory disease models, including asthma, inflammatory bowel disease, and experimental autoimmune encephalomyelitis.[10–12] Herein, we investigated the connection between eosinophils and natural killer (NK) cells, highlighting a new pathway responsible for the control of eosinophilic accumulation in sites of inflammation.

Our group and others have reported that NK cells, an innate lymphoid cell subset, are protective in coxsackievirus B3 and murine cytomegalovirus animal models of myocarditis by limiting viral replication.[13–15] Because myocarditis is also an autoimmune-mediated disease, it is unknown if NK cells can protect against disease through limiting viral replication, as well as by reducing the autoimmune response.[16,17] The data regarding NK cells and autoimmunity are extensive, but conflicting. NK cells accumulate in joints during rheumatoid arthritis (RA), skin lesions during psoriasis, and brain lesions during multiple sclerosis.[18,19] Activated NK cells from the joints of RA patients induce differentiation of monocytes, signifying an active role in the immune environment,[20] and indicating that NK cells play a proinflammatory role in autoimmunity.

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This directly contradicts the observations that myocarditis, RA, Sjögren syndrome, and systemic lupus erythematosus patients have decreased NK cell numbers and cytotoxicity potential.[21–25] A limited study of biopsy specimens from myocarditis patients revealed a lack of NK cells in the cardiac tissue.[26] Peripheral NK cells from RA patients failed to induce apoptosis in major histocompatibility complex I–deficient K562 cells versus healthy controls *in vitro*. Patients with multiple sclerosis in remission had higher frequencies of activated peripheral NK cells than those with active disease, supporting the notion that defects in NK cells are associated with increased risk of autoimmunity.[27] Altogether, it is unclear whether autoimmune diseases are exacerbated by deficiencies or excesses of NK cells, making animal studies necessary.

Herein, we investigated the role of NK cells in autoimmune myocarditis using a mouse model of experimental autoimmune myocarditis (EAM). EAM is induced by s.c. immunization of myocarditogenic peptide in complete Freund's adjuvant, the same antigen targeted by autoaggressive T cells in coxsackievirus group B type 3 (CB3)-induced myocarditis.[28–31] Susceptible mice strains develop myocarditis, followed by inflammatory dilated cardiomyopathy.[29] EAM induces the immune response independent of persistent virus, allowing us to separate autoimmune- from virus-mediated disease. We report herein the ability of NK cells to control myocarditis in the absence of a viral pathogen.

Materials and Methods

Mice

BALB/c, *Rag1*$^{-/-}$, C.Cg-*Gata1*^{tm6Sho}/J (ΔdoubleGATA1), CD3δ-IL5Tg NJ.1636, *Ccr3*$^{-/-}$, interferon γ receptor 1 (IFNγR1)$^{-/-}$, and IFNγ$^{-/-}$ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were bred and maintained in the conventional housing facilities at Johns Hopkins University (Baltimore, MD). All protocols have been reviewed and approved by the Johns Hopkins Animal Care and Use Committee.

Immunization with $MHC_{614-629}$ and Assessment of EAM

Male 6- to 8-week-old BALB/c mice were injected s.c. with 100 μg of myocarditogenic peptide of cardiac myosin heavy chain (MyHC), $MyHC_{614-629}$, emulsified in an equal volume of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO), supplemented with 4 mg/mL of H37Ra extract (Difco, Lawrence, KS) on days 0 and 7, as previously described.[29] Pertussis toxin (500 ng in 100 μL of phosphate-buffered saline (PBS; Sigma-Aldrich) was administrated i.p. on day 0. Mice were sacrificed on day 21; hearts were collected and sections were stained with hematoxylin and eosin, as previously described.[32–35] The degree of myocardial infiltration and fibrosis was determined blindly by two individuals (D.C. and S.O.), and histology was scored as follows: 0, no infiltration; 1, ≤10%; 2, 11% to 30%; 3, 31% to 50%; 4, 51% to 90%; and 5, >90%.[29]

Assessment of Fibrosis

Mice were sacrificed on day 21. Hearts were collected and sections were stained with Masson's trichrome, as previously described.[32–35] Images of Masson's trichrome–stained cardiac sections were uploaded into ImageJ software version 1.48 (NIH, Bethesda, MD). The background space was deleted and the left ventricle, the region of interest, was selected using the freeform loop tool. Pixels within the selected area were deconstructed into red (tissue) or blue (collagen) channels, and fibrosis was calculated as a percentage of blue versus total red plus blue pixels in the region of interest.

Echocardiography

An Acuson Sequoia 256 high-resolution microimaging system with a 13-MHz transducer was used (Visualsonic, Toronto, ON, Canada). In conscious mice, the heart was imaged in the two-dimensional mode in the parasternal short-axis view. From the M-mode, the left ventricular (LV) wall thickness and chamber dimensions were measured. The M-mode cursor was positioned perpendicular to the intraventricular septum and the LV posterior wall, with three to five readings taken for each measurement. The LV end diastolic dimension, LV end systolic dimension, LV posterior wall thickness at end diastole, and the intraventricular septal wall thickness at end diastole were measured from a frozen M-mode tracing. Fractional shortening, ejection fraction, and relative wall thickness were calculated as previously described.[32]

Intracardiac and Splenic Flow Cytometry

The aorta was cannulated to perfuse hearts with 15 mL of cold 1× PBS for 3 minutes to remove blood. To generate cardiac single-cell suspensions, hearts were bisected, placed in C-tubes, and dissociated on the GentleMACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) under program heart_01. Cells were placed in a rotating incubator with 10 mg of collagenase II and 1.5 mg of DNase I (Worthington Biochemical, Lakewood, NJ) for 30 minutes at 37°C. Cells were dissociated again and rinsed twice with 1× PBS with 0.05% bovine serum albumin (BSA; Sigma-Aldrich) and 2 mmol/L EDTA (Corning Cellgro, Corning, NY). To generate a splenic single-cell suspension, spleens were dissociated between two frosted glass slides and incubated with 2 mL of ACK Lysing Buffer for 1 minute. The cells were rinsed with 1× PBS (Mediatech, Manassas, VA) and filtered through a 40-μm mesh. Cells (1 to 3 × 10^6) were incubated with 1 μL of LIVE/DEAD Aqua (Invitrogen, Carlsbad, CA) for 30 minutes in 1× PBS to stain