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The response of aged mice to primary infection and re-infection with pneumonia virus of mice depends on their genetic background

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ABSTRACT

The pneumonia virus of mice (PVM) model is used to study respiratory syncytial virus (RSV) pathogenesis. The outcome of PVM infection varies in different inbred mouse strains, BALB/c being highly susceptible and C57BL/6 more resistant. As the disease symptoms induced by RSV infection can become more severe as people age, we examined the primary and secondary immune responses to infection with PVM in aged BALB/c and C57BL/6 mice. Based on clinical parameters, aged C57BL/6 mice displayed less severe disease than young adult mice when infected with 3000 pfu of PVM-15, while BALB/c mice were equally susceptible at both ages showing significant weight loss and high levels of virus replication. Furthermore, after primary infection the $CD4^+$ T cell numbers in the lungs were higher in young adult mice, while the $CD8^+$ T cell numbers were comparable in both age groups and strains. When either C57BL/6 or BALB/c mice were infected with PVM as young adults and then re-infected as aged mice, they were protected from clinical disease, while virus replication was reduced. In contrast to mice with a primary PVM-infection, re-infected mice did not have infiltration of neutrophils or inflammatory mediators in the lung. BALB/c mice had higher virus neutralizing antibody levels in the serum and lung than C57BL/6 mice upon re-infection. Re-infection with PVM led to significant influx of effector $CD4^+$ T cells into the lungs when compared to aged mice with a primary infection, while this cell population was decreased in the lung draining lymph nodes in both mouse strains. After re-infection the effector $CD8^+$ T cell population was also decreased in the lung draining lymph nodes in both mouse strain when compared to aged mice after primary infection. However, the central memory $CD4^+$ and $CD8^+$ T cells were significantly enhanced in numbers in the lungs and draining lymph nodes of both mouse strains after re-infection, and these numbers were higher for C57BL/6 mice.

1. Introduction

Human respiratory syncytial virus (RSV) is a single-stranded, negative sense, highly transmissible RNA virus causing infant mortality and hospitalization worldwide (Glezen and Denny, 1973; Meng et al., 2014; Zhou et al., 2012). Primary RSV infections are poorly protective leading to reinfections throughout life. In healthy adults and children re-infections are self-limiting; however, in infants, young children, immune-compromised and elderly individuals, severe RSV complications can occur resulting in respiratory failure and death (Hall et al., 1991; Simoes, 1999). It has been reported that in the USA RSV infections result in an estimated 10,000 deaths per year in adults over the age of 65 (Cherukuri et al., 2013; Wong et al., 2014). The factors involved in the development of severe RSV illness in elderly are largely unknown. However, declined and repressed T cell responses against RSV in elderly may contribute to the severity of re-infections (Busse and Mathur, 2010; Fulton and Varga, 2009; Johnstone et al., 2008; Wong et al., 2014).

Owing to the limitations associated with studies involving human subjects with RSV infection and to the fact that RSV replicates poorly in mice, pneumonia virus of mice (PVM), a rodent pathogen, has been evaluated for the past three decades as a potential murine model to understand severe pneumovirus infection in an experimental host system (Bem et al., 2011; Dyer et al., 2012). PVM replicates in the lung tissue of all inbred strains of mice; however, the degree of inflammatory responses to PVM depends on the mouse strain (Dyer et al., 2012). Genetic differences between

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BALB/c and C57BL/6 mice can lead to very different responses following natural PVM infection *in vivo* (Watkiss et al., 2013). In addition, BALB/c mice have the H-2d locus while C57BL/6 have the H-2b locus, similar to differences among human populations and the disease severity in RSV infection being associated with MHC haplotype (Tregoning et al., 2010). We have previously used the PVM model to explore the pathogenesis of PVM-15 in BALB/c and C57BL/6 mice and showed that the difference in susceptibility of these two strains might be due to the capacity of the C57BL/6 mice to control both the viral replication and the immune response elicited by PVM (Watkiss et al., 2013).

Aging is associated with a progressive decline in both innate and adaptive immunity (Kumar and Burns, 2008; Larbi et al., 2011; Lee et al., 2012). Our understanding of the senescent immune system and the use of the aged mouse model to study age-related changes in the response to RSV infection is still in its infancy. Not much is known about RSV-specific immunity in aging populations and the role of immunosenescence in RSV pathogenesis (Cusi et al., 2010; Fulton et al., 2013; Liu and Kimura, 2007; Mosquera et al., 2014). In an attempt to understand the effects of aging on the immune system in context of PVM infection as a model for RSV, we examined innate and adaptive immune responses, as well as the protective efficacy of these responses upon re-infection in BALB/c and C57BL/6 mice. This study gives us an insight into the effect of the aging immune system on the outcome of PVM infection and whether it mimics RSV re-infection conditions.

2. Materials and methods

2.1. Cell lines, virus stock and infection of mice

PVM-15 was propagated in Baby Hamster Kidney (BHK)-21 cells and the viral titre was determined as described previously (Watkiss et al., 2013). Six to eight week-old BALB/c and C57BL/6 mice (Charles River Laboratories) were housed in groups of four to six and acclimatized for one week before challenge. Adult mice were inoculated intranasally with 50 μl of medium or with 300 pfu of PVM-15 in 50 μl under light anaesthesia. Mice were re-infected at 64 weeks of age by intranasal inoculation of 3000 pfu PVM-15. Age-matched mice obtained a primary infection with an equivalent amount of PVM-15. Mice were weighed daily and scored for clinical illness according to a modified version of Morton and Griffiths (1985). Sera were collected at regular intervals for detection of IgG. 10–12 adult and aged mice per group were euthanized on day 6 after primary and re-infection with PVM-15 to collect lungs, bronchoalveolar lavage (BAL) fluids and splenocytes. Mice were bled out by cardiac puncture. All experiments were performed within the guidelines of the Canadian Council for Animal Care.

2.2. Collection of lung samples and cytospins

Lung samples were collected as described previously (Watkiss et al., 2013). Briefly, the single-lobed, left lung was clamped off and homogenized for cytokine multiplex assay. The multi-lobed, right lung was collected into Trizol® reagent (Invitrogen) for RNA isolation. All samples were flash-frozen in liquid nitrogen immediately following centrifugation, and stored at −80 °C. Bronchoalveolar lavage fluid was collected prior to removal of the right lung by washing it with 500 μl of phosphate buffered saline (PBS), pH 7.2 (Invitrogen) supplemented with 2% FBS and 50 μM EDTA. Fluids from groups of four to six mice were pooled, and 5×10^4 and 1×10^5 BAL cells per slide were used to prepare cytospin slides with a Cytospin 4 (Thermo Shandon). The slides were stained with a Giemsa–Wright stain (Bayer HealthCare) using an automated slide stainer, and differential analysis of the cell populations was performed by counting at least 200 cells in a blinded manner.

2.3. Cell isolation and flow cytometry

Lung cell isolation was carried out as previously described (Watkiss et al., 2013). Perfused lungs were excised and disrupted by tissue dissociation using a GentleMACS machine (Miltenyi Biotec) and incubated with collagenase type IA (0.5 mg/ml; Sigma–Aldrich) and type IV bovine pancreatic DNase (20 μg/ml; Sigma–Aldrich) for 30 min at 37 °C. Lung cells were filtered through 70 μm cell strainers (BD Falcon), washed and then treated with ACK lysis buffer (Invitrogen). Cell viability was assessed by trypan blue exclusion, and total cell numbers were determined using a Beckman Coulter counter. For assessment of lung DC populations CD11c cells were isolated using CD11c microbeads (Miltenyi Biotec). Mediastinal lymph nodes (MLNs) were harvested, pooled, minced and passed thrice through cell strainers, washed and resuspended in medium. Cell viability and total cell numbers were assessed.

Prior to staining cells were blocked with CD16/32 (Fc block; BD Pharmingen). For surface staining, the following antibodies were used; anti-CD3 (cat no. 555275), anti-CD4 (cat no. 553729), anti-CD8 (cat no. 553031), and anti-MHCII (cat no. 553610) (BD Pharmingen), anti-CD11c (cat no. 117308), anti-CD11b (cat no. 101205), and anti-CD45R/B220 (cat no..103205) (BioLegend); and m-PDCA-1 (5120327174) (Miltenyi Biotec). To identify the memory phenotype, single cell suspensions from lung and MLNs were stained with FITC anti-CD8, CD4 (BD Pharmingen), APC anti-CD127 and PerCP-Cy5.5 anti-CD62L (BioLegend). Cells were gated for live cells, singlets and lymphocytes and then analyzed. Flow cytometry was performed using a FACS Calibur (BD Biosciences). Analysis of flow cytometry data was performed with Kaluza software (Beckman Coulter).

2.4. Cytokine multiplex assay

Cytokines and chemokines were quantified in the cell-free supernatants of lung homogenates using the electrochemiluminescence (ECL) detection-based meso scale discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg) according to the manufacturer's instructions. The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations based on several log calibrator curves.

2.5. Lung fragment cultures, PVM quantification and PVM neutralization assay

Lung fragment cultures (LFC) were prepared and PVM quantification and neutralization was carried out using a standard immunofluorescent plaque assay as previously described (Watkiss et al., 2013).

2.6. Enzyme-linked immunosorbent assay

For detection of anti-PVM antibodies in multiple sequential serum samples a SMART-M12 kit (Biotech Trading Partners) was used as per manufacturer's instructions (Bondue et al., 2011). PVM-specific IgG and IgA were evaluated in LFC by ELISA as described previously (Watkiss et al., 2013). Briefly, Immulon II plates (Thermo Electron) were coated with PVM-infected and mock-infected cell lysates, washed, and blocked with 5% gelatin in PBS (Sigma–Aldrich). Subsequently, dilutions of serum or LFC supernatant were prepared, added to the coated ELISA plates and incubated overnight at 4 °C. After washing, alkaline phosphatase (AP)-conjugated IgG (Kirkegaard & Perry Laboratories) or alternatively, biotinylated-anti-mouse IgA (Invitrogen) followed