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Immunogenic Dendritic Cells Primed by Social Defeat Enhance Adaptive Immunity to Influenza A Virus

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Abstract

Dendritic cells (DCs) sample their surrounding microenvironment and consequently send immunogenic or regulatory signals to T cells during DC/T cell interactions, shaping the primary adaptive immune response to infection. The microenvironment resulting from repeated social defeat increases DC co-stimulatory molecule expression and primes DCs for enhanced cytokine responses *in vitro*. In this study we show that social disruption stress (SDR) results in the generation of immunogenic DCs, capable of conferring enhanced adaptive immunity to influenza A/PR/8/34 infection. Mice infected with influenza A/PR/8/34 virus 24h after the adoptive transfer of DCs from SDR mice had significantly increased numbers of D^bNP₃₆₆₋₇₄CD8⁺ T cells, increased IFN- γ and IFN- α mRNA, and decreased influenza M1 mRNA expression in the lung during the peak primary response (9 days post-infection), compared to mice that received DCs from naïve mice. These data demonstrate that repeated social defeat is a significant environmental influence on immunogenic DC activation and function.

Introduction

Dendritic cells (DCs) are known to have potent, but differential, immunoregulatory capabilities, dependant upon the environment that they are sampling (Banchereau et al., 2000; Steinman and Hemmi, 2006). Regulatory or tolerogenic DCs, which enhance Th2 or regulatory T cell responses, can be generated after exposure to hormones, cytokines, or soluble mediators like glucocorticoids (CORT), vitamin D, IL-10, or TGF- β (Adler and Steinbrink, 2007). In contrast, immunogenic DCs, which propagate inflammatory, Th1, and Th17 responses, can be generated after exposure to inflammatory signals like LPS (Cella et al., 1997; De Smedt et al., 1996), IL-1 β (Wesa and Galy, 2001), or ligation of CD40 on the cell surface (Cella et al., 1996). The release of neuroendocrine hormones (e.g. CORT and catecholamines) and circulating cytokines (e.g. IL-1, TNF- α and IL-6) as a consequence of stress alters the milieu in which DCs are primed, activated, and take up, process, and present

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antigen (Bros et al., 2007; Maestroni, 2006; Sabatte et al., 2007; Saint-Mezard et al., 2003). Stress induced changes in the microenvironment influence DC priming and activation, which will, in turn, further regulate and shape the adaptive immune response.

The activation of naïve CD8⁺ T cells depend on antigen presentation by DCs. Several factors contribute to the efficacy of DC-T cell interactions including the number of cells present, the concentration of antigen, and, importantly, the expression of cell surface molecules (e.g. MHC, CD80, CD86, and cell adhesion molecules) (Bousso and Robey, 2003; Guernonprez et al., 2002; Hall et al., 2004; Moser and Murphy, 2000). Upon activation, T cells migrate to and enter damaged, inflamed, or infected tissue. CD8⁺ T cells eliminate and maintain immunity to influenza virus infection. These cells facilitate the clearance of influenza virus-infected cells by secretion of cytolytic agents (e.g. perforin and granzymes) and cytokines, (e.g. IFN- γ), and through Fas/Fas ligand mediated apoptosis (Doherty et al., 1997; Lukacher et al., 1984; Topham et al., 1997). After termination of the primary viral infection, memory CD8⁺ T cells provide long-lasting protection against re-infection with closely related virus strains (Woodland et al., 2001).

Previous studies from our laboratory indicate that neuroendocrine activation, through the experience of social disruption stress (SDR), or repeated social defeat, results an increase in co-stimulatory molecules important for CD8⁺ T cell activation (e.g. CD80, MHC I, CD44) on the surface of CD11c⁺ DCs from SDR mice (Powell et al., 2009). Further, SDR enhances the adaptive response to influenza A/PR/8/34 virus infection, and increases the size of the immunodominant memory D^bNP₃₆₆₋₇₄CD8⁺ T cell subset specific for the NP 366-74 epitope of the A/PR/8/34 virus (Mays et al., 2010). NP 366-74 is an immunodominant epitope that stimulates a CD8⁺ T cell response and is critical for establishing a memory response to influenza A/PR/8/34 virus infection (Belz et al., 2001). A possible mechanism for the stress-enhanced adaptive responses to influenza A infection is the stress-induced priming of DCs, leading to more effective antigen presentation. This study was designed to determine the impact of DCs, primed in an SDR-induced microenvironment, on the development of an influenza-specific, D^bNP₃₆₆₋₇₄CD8⁺ T cell response. These data showed that the experience of repeated psychosocial stress resulted in the priming of immunogenic DCs and translated to enhanced adaptive immune responses to an influenza A/PR/8/34 virus infection.

Materials and Methods

Mice

Male C57BL/6 mice between the ages of 6–8 weeks were purchased from Charles River Laboratories (Wilmington, MA) and allowed to acclimate to the animal facility for 1 week prior to any procedures. Mice were housed in groups of 3 per cage and were maintained on a 12 h light/dark schedule with the lights on at 0600 h in an AAALAC (American Association of Accreditation of Laboratory Animal Care) accredited facility. Food and water were available *ad libitum*, and all procedures with animals were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee (ILACUC).

Social Disruption Stress (SDR)

SDR was employed as previously described (Avitsur et al., 2005; Bailey et al., 2007; Powell et al., 2009). Briefly, aggressive intruder male mice were introduced into cages of established cohorts of three male mice for 2h between 1630 and 1830 for six consecutive nights. At the end of the 2h period, the aggressor was removed and the residents were left undisturbed until the following day when SDR was repeated. Home cage control (HCC) mice were housed in a separate room and left undisturbed until the day of cell harvest.

Influenza peptide and tetramer reagents

The NP366-74 (influenza) peptide (ASNENMETM) was synthesized by The OSU Peptide Synthesis Facility (Columbus, Ohio). The NP366-74 tetramer probe consists of four peptide/H-2Db complexes conjugated to streptavidin-PE. The tetramer probes were conjugated by the NIH Tetramer Facility at Emory University (Atlanta, GA).

Influenza viral infection

Mice were infected intranasally 24h after saline injection (no DC control group) or the transfer of CD11c⁺ DCs from SDR or home cage controls (HCC). Frozen stocks of influenza A/PR/8/34 virus, grown in the allantoic fluid of embryonated chicken eggs as previously reported (Mays et al., 2010), were used. Immediately prior to infection, mice were anesthetized by intramuscular injection of Ketamine/Xylazine (78.1/4.4 mg/kg, Vedco, St. Joseph's, MO) diluted in sterile PBS. A 50% lethal dose for this virus, 0.1 hemagglutinating unit (HAU) of influenza A/PR/8/34 suspended in 50 µl sterile PBS, was used for infection.

Flow cytometry

Single cell suspensions derived from HCC or SDR spleen tissue (0.5×10^6 cells per sample) were incubated with 1 µg each of fluorescently-labeled monoclonal antibodies (or the appropriate isotype controls) after a 20-min blocking step with Fc receptor block (BD Biosciences, San Jose, CA). Antibody labeling was performed at 4°C for 30 min. The cells were then washed twice in PBS containing 1% FBS and 0.09% NaN₃ and fixed with 1% paraformaldehyde. Cytotoxic T cells (CD3⁺, CD8⁺ cells) were identified using fluorescein (FITC)-labeled anti-CD3e antibody and allophycocyanin (APC)-labeled anti-CD8a (Ly-2) antibody (BD Biosciences, San Jose, CA). Influenza-specific CD8⁺ cells (NP₃₆₆₋₇₄Tetramer⁺/CD3⁺/CD8⁺ cells) were identified by gating on the CD3⁺/CD8⁺ lymphocyte population and then selecting the CD8⁺/NP₃₆₆₋₇₄⁺ events. Lymphocytes were gated based on forward versus side scatter and a total of 10,000 events were analyzed. Data were collected with a FACSCalibur flow cytometer and analyzed with Cell Quest Pro (BD Biosciences, San Jose, CA).

Adoptive transfer and enrichment of CD11c⁺ and CD8⁺ cells

Following the 6th cycle of SDR, mice were anesthetized and spleens were removed from each group (SDR or HCC) of animals and single cell suspensions were prepared as described previously (Powell et al., 2009). DCs from SDR or HCC mice were selected from cell suspensions using Pan DC Microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. CD8⁺ T cells for *in vitro* co-cultures were obtained from influenza infected mice and were positively selected from cell suspensions using CD8⁺ T cell Microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. Flow cytometric analysis showed greater than 96% purity in each positive fraction. For transfer experiments, approximately 1×10^6 DCs from either the SDR or HCC mice were transferred intravenously (i.v.) to naïve, non-stressed recipients. To determine the ability of DCs to traffic to the lung of recipient mice, DCs were labeled with the fluorescent dye CFSE prior to transfer. 24h after the transfer of CFSE-labeled DCs, lungs of recipient mice were removed and the percentage of CFSE-labeled DCs was measured in SDR DC and HCC DC recipient groups by flow cytometric analysis. This percentage of total CFSE⁺ cells in the lung did not differ between mice receiving SDR (8.86% $\pm$ 1.3 of total cells) or HCC DCs (8.79% $\pm$ 0.9 of total cells), indicating that DCs from both SDR and HCC mice are equally able to traffic to the lung. Approximately 37.7% ($\pm$ 2.76) of transferred SDR DCs and 40.7% ($\pm$ 5.27) of transferred HCC DCs from donor mice were recovered in the lung of recipients 24h after transfer.

Real-time PCR

RNA was isolated from the apical lobe of the lung in TRIzol according to manufacturer's instructions (GibcoBRL, Rockville, MD). Reverse transcription and cDNA synthesis were carried out using a Promega kit (Madison, WI). Cytokine primer and probe sequences were developed with Primer Express Software from PE Biosystems (Foster City, CA.), and the influenza A matrix (M1) protein, IFN- γ , and IFN- α primers and probes were created using previously published sequences (Hughes et al., 1994; Lin et al., 2006; van Elden et al., 2001).

Cytometric bead array

CD8⁺ T cells were isolated and were cultured in a 1:5 DC:CD8⁺ T cell ratio in 96-well plates together with NP366-74 peptide (10 μ g/ml) or medium, and DCs from SDR or HCC mice. As described previously (Powell et al., 2009), supernatants were harvested and cytokine analyses performed using the BD inflammation cytometric bead array according to manufacturer's protocol (BD Biosciences, San Jose, CA). Data for cytokines not present in supernatants or not significantly different between groups were not shown.

Statistical Analysis

Results are reported as mean $\pm$ SEM. The data were analyzed by a Student's *t* test or a mixed parameter ANOVA with a post hoc *t* test with Bonferroni correction. Groups were considered statistically significant at $p < 0.05$.

Results

Previous studies in our laboratory demonstrated that SDR enhances the innate and adaptive immune response to infection (Bailey et al., 2007; Dong-Newsom et al., 2009; Mays et al., 2010; Powell et al., 2009). Specifically, DCs from mice experiencing repeated social defeat are primed for enhanced cytokine secretion, insensitive to CORT-induced cell death, and express increased levels of co-stimulatory molecules on their surface (Powell et al., 2009). In addition, SDR lead to enhanced antigen-specific CD8⁺ T cell memory (Mays et al., 2010). In order to determine whether DCs from SDR mice could confer enhanced antigen-specific CD8⁺ T cell immunity to an influenza viral infection, purified CD11c⁺ DCs from SDR or HCC mice were transferred to non-stressed, naïve recipients, or naïve mice were treated with vehicle alone. Approximately 24h post transfer or vehicle treatment, recipient mice were intranasally infected with influenza A/PR/8/34 virus. T cell number from the lung of infected mice in each group was assessed at day 9 p.i. by flow cytometry (Fig. 1 A–E). Lung cellularity 9 days p.i. was significantly increased ($p < 0.01$) in SDR DC recipients when compared to HCC DC recipients (Fig. 1A). After flow cytometric analysis of cell phenotype infiltrating the lung tissue during infection, it was evident that there was a significant increase in total lymphocytes (Fig. 1B; $p < 0.01$) as well as CD8⁺ T lymphocytes (Fig. 1C; $p < 0.02$) in the lung. Importantly, there was a significant increase in the DbNP₃₆₆₋₇₄CD8⁺ T cell population (Fig. 1D–E; $p < 0.001$).

DCs are necessary for antigen presentation to naïve CD8⁺ T cells during an influenza infection and are a critical cell in activating anti-viral cytotoxic T cell responses that limit viral replication. In order to determine if the transfer of SDR DCs had an impact on viral replication in lung tissue, gene expression of the matrix protein (M1) of A/PR/8/34 virus in the lung of affected mice was assessed by real-time RT-PCR. There was a significant fold-decrease in M1 gene expression in mice pre-treated with SDR DCs (Fig. 2A) when compared to the control group ($p < 0.01$). Thus, viral replication was terminated earlier by pre-treatment with SDR DCs.

Nine days p.i., M1 gene expression negatively correlated with a significant increase in epitope-specific CD8⁺ T cells in the lungs of mice that received SDR-DCs prior to infection. Levels of IFN- α and IFN- γ mRNA were also measured in the lung by real-time RT-PCR, 9 days p.i. in mice receiving no DCs, HCC DCs, or SDR DCS. Along with increased T cell infiltration into the lung and increased M1 gene expression, there were significant increases in IFN- α (Fig. 2B; $p < 0.04$) and IFN- γ (Fig. 2C; $p < 0.05$) gene expression in the lungs of mice receiving SDR DCs.

Companion cultures of DCs from HCC or SDR mice alone, or co-cultured with CD8⁺ T cells from influenza infected mice were stimulated with the NP 366-74 peptide (10 μ g/ml). The total amount of IFN- γ from the DC cultures alone was considered baseline, and was subtracted from the stimulated groups during analysis. Cultures containing SDR DCs and CD8⁺ T cells showed a significant increase in the amount of IFN- γ when compared with cultures containing HCC DCs and CD8⁺ T cells (Fig. 3; $p < 0.05$). Cultures containing peptide stimulated SDR DCs alone or HCC DCs alone without T cells did not result in elevated or different levels of IFN- γ in the supernatants (data not shown).

Prior to the adoptive transfer of DC populations from SDR or HCC mice to naïve recipients, the phenotype (Fig. 4A–B) and activation status of DCs from each group were analyzed by flow cytometry. Consistent with the findings reported in Powell et. al 2009, DCs from SDR mice in the current study showed a significant increase in the expression of CD80, MHC I, and CD44 on their surface when compared to DCs from HCC mice (data not shown). In addition, the number of DCs from SDR and HCC mice was not different, although the spleen size in SDR mice was significantly increased (Fig. 4C; $p < 0.001$). The majority of DCs in both SDR and HCC groups were of the conventional phenotype, with approximately 75% of the total CD11c⁺ cells co-expressing CD11b (Fig. 4A). A smaller subset, approximately 25% of the total CD11c⁺ cells were also B220⁺, consistent with a plasmacytoid phenotype (Fig. 4B).

Discussion

Previous reports from our laboratory show that enhanced innate and adaptive immune responses to viral infection are evident after exposure to repeated social defeat (Dong-Newsom et al., 2009; Mays et al., 2010). These new data provide evidence that SDR primes DCs for enhanced immunogenic responses, and the transfer of SDR-primed immunogenic DCs alone increases the number of D^bNP₃₆₆₋₇₄CD8⁺ T cells, reduces viral replication, and enhances cytokine secretion in the lung as well as in antigen specific CD8⁺ T cells after an influenza A virus infection. This directly links the experience of a stressful social interaction to the enhancement of specific immune cell function in the context of a viral infection. Further, these data demonstrate that stress can mediate its effects on enhanced adaptive immunity to viral infection, in part, through the priming and activation of immunogenic DCs.

These data show that DCs from SDR mice directly increase the number and enhance the cytokine secretion of CD8⁺ T cells in an antigen specific manner. This increase in D^bNP₃₆₆₋₇₄CD8⁺ T cells at the site of infection carries both negative and positive implications. Efficient lung function is dependent upon lung architecture, and an increase in cellularity may result in reduction in available space for gas exchange (Bruder et al., 2006; Pascual and Peters, 2005). Further, hypercytokinemia is one of the major factors behind influenza-related morbidity and mortality (Bermejo-Martin et al., 2009; Lee et al., 2007). After resolution of a primary influenza infection, increased residual damage was observed by histopathological analysis in lung tissue of SDR-experienced mice versus that of non-stressed infected mice (Mays et al., 2010). Additionally, reports by Curry et al. indicate that

SDR, in the absence of infection, can cause a significant increase in the expression of the adhesion molecules P-selectin, E-selectin, and ICAM-1 on lung epithelium and a significant enhancement of chemokine mediated recruitment of neutrophils and monocytes to the lung (Curry et al., 2009). Stress-induced expression of lung specific chemotactic factors and adhesion molecules on lung epithelium, coupled with increased primed cell subset will promote increased cellular traffic to the lung. An increase in cellularity can lead to robust local inflammatory responses and virus-associated lung epithelial damage during influenza infection. On the other hand, more cells may predict a faster and more efficient resolution of disease.

The amount of A/PR/8/34 virus M1 mRNA in infected tissue during viral replication is indicative of the amount of virus present in tissue and is detectable until virus replication ceases and virus is gone from the tissue. M1 gene expression, therefore, serves as a surrogate marker of influenza viral replication in infected tissue (Ali et al., 2000; Liu and Ye, 2002). Stress has been shown to alter viral replication in various disease systems. Multiple studies have shown that chronic stress, restraint stress (RS) in particular, induces a generalized immunosuppression and a permissive state for viral replication and spread. Stress-induced changes include decreased infiltration of leukocytes to the site of infection, decreased virus-specific cytokine secretion, and dampened antigen-specific T cell responses (Bonneau et al., 1991; Mi et al., 2006; Sheridan et al., 1991; Wonnacott and Bonneau, 2002). Mice exposed to repeated social defeat, unlike those exposed to RS, display enhanced immune reactivity as indicated by increased number, activation, and function of immune cells. Several autoimmune and inflammatory disease conditions are known to increase in severity after exposure to stress (Hildebrand et al., 2000; Mohr et al., 2000; Vig et al., 2006). Additionally, depending upon the nature and the duration of the stressor, innate and adaptive immune function can be enhanced after exposure to stressful stimuli.

Stress hormones such as glucocorticoids and catecholamines are known to regulate immune cell function, particularly at the level of cytokine secretion (Angeli et al., 1999; Johnson et al., 2005). Cytokines provide critical signals for both the activation of antigen-specific naïve cells and propagation of immune cell function in response to viral infection. During primary viral infections, type I interferons (IFN α/β) serve to restrict the spread of the virus by inducing an antiviral state in uninfected cells and triggering apoptotic events in infected cells. Primary sources of the type I interferons during pulmonary infections are virus infected epithelial cells, macrophages and plasmacytoid DCs (Julkunen et al., 2001). In addition, it has been shown that conventional CD11b⁺/CD11c⁺/B220⁻ DCs can also secrete high levels of IFN- α after viral infection (Diebold et al., 2003). Secretion of IFN- γ by virus specific CD8⁺ T cells is an important mechanism for the inhibition of viral replication and viral clearance (Wiesel et al., 2009) IFN- γ stimulates CTL mediated killing, increases MHC I expression, activates macrophages and neutrophils, and promotes Th1 cell proliferation (Boehm et al., 1997). While some stressors have been shown to reduce pro-inflammatory and antiviral cytokine secretion, SDR enhances antiviral and inflammatory cytokine secretion (Mays et al., 2010; Meagher et al., 2007). Our study suggests that the SDR-induced increase in IFN- α and IFN- γ levels was facilitated, at least in part, by primed DCs. Although the number of conventional and plasmacytoid DCs did not change after SDR, a closer look at the function of conventional and plasmacytoid DCs after repeated social defeat is required to determine if SDR-induced increased IFN- α and IFN- γ production is directly linked to alterations in functional aspects of DC sub-populations.

In addition to the significant changes noted when compared to SDR DC treated mice, the transfer of HCC DCs resulted in a slight, but not significant, decrease in cytokine responses and cellularity when compared to animals receiving no DC treatment at all prior to influenza A/PR/8 infection. This was not a surprising result as subsets of DCs, while phenotypically

similar, can be functionally distinct. This is particularly true for tolerogenic or regulatory DCs (e.g. suppressive TGF- β /IL-10 producing DCs) when compared with immunogenic or conventional DCs (e.g. inflammatory IFN- γ /IL-12 producing DCs) (Banchereau et al., 2000; Shortman and Naik, 2007). DCs coming from a steady-state environment tend to be in an immature (or semi-mature) state and have a more regulatory function (Mahnke et al., 2002), whereas cells coming from the SDR environment appear to be primed and have enhanced immunogenic function. The transfer of DCs from a naïve mouse with a tendency toward regulatory or tolerogenic function would confer decreased inflammation to the recipient mouse. In the case of SDR DCs, the SDR environment is imprinted on the DCs prior to transfer into the naïve environment of the recipient host and that SDR-imprinted DC is capable of transferring the enhanced immune responses, such as enhanced influenza-specific T cell responses as seen in evident in the SDR mouse (Mays et al., 2010).

The decrease in M1 gene expression and increase in antiviral cytokine gene expression in mice receiving DCs from SDR mice prior to A/PR/8/34 infection shows the importance of DC activation and priming prior to an infection or vaccination. It is known that DCs play a crucial role in setting up and maintaining the immune response to influenza A. In particular, the functional capacity of DCs as well as the maturation status and phenotypic subset of DCs infiltrating the lung during an influenza-specific response directly effect CD8⁺ T cell priming and function (Ballesteros-Tato et al., 2010; GeurtsvanKessel et al., 2009; Larsson et al., 2000). Additionally, the environment in which a DC is primed has direct consequences on the ability of DCs to mount significant antigen-specific CD8⁺ T cell responses to influenza A/PR/8/34 (Smith et al., 2009). Further, DCs can both directly and indirectly suppress viral replication by enhanced anti-viral cytokine secretion or by effectively stimulating anti-viral CD8⁺ T cells.

This study demonstrates the impact that stressful social interactions and stressful stimuli have on immune cell function. The experience of repeated social defeat leaves a significant imprint on DCs, as the immune consequences of a stressful social interaction can be transferred by DCs from a stressed donor to a non-stressed recipient. Specifically, when DCs were taken from an environment of repeated social defeat and transferred to a non-stressed recipient, the enhanced antiviral immune responses previously observed in donor mice was conferred to the non-stressed recipient of the SDR-primed DCs. The transfer of SDR-primed DCs ultimately resulted in more effective adaptive immune responses in recipient mice, implicating DCs as a primary mediator of the regulatory effects of stress on the adaptive immune response to infection. Delineating the underlying behavioral and biological mechanisms that lead to the generation of immunogenic DCs has significant clinical implications for the treatment and prevention of influenza A virus infection, as well as the regulation of CD8⁺ T cell responses.

- SDR, a model of social defeat, significantly influences immunogenic DCs
- Adoptively transferred DCs from SDR mice enhance adaptive immunity to influenza
- SDR DC recipients have increased D^bNP₃₆₆₋₇₄CD8⁺ T cells in the lung after infection
- SDR DC recipients have increased IFN- α and IFN- γ mRNA in the lung after infection
- SDR DC recipients have decreased M1 mRNA in the lung after infection

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Figure 1. The frequency of cells, including antigen specific CD8⁺ T cells, was significantly increased in the lung of influenza infected mice that received SDR DCs prior to infection Lungs were removed from influenza infected mice receiving no DCs, HCC DCs, or SDR DCs. The proportion of (A) total cells (* $p < 0.01$), (B) total lymphocytes (* $p < 0.01$), (C) total CD8⁺ T cells ($p < 0.02$), and (D–E) total NP₃₆₆₋₇₄CD8⁺ T cells (* $p < 0.001$) detected at day 9 of influenza infection was affected by treatment with SDR DCs. Data represent three separate experiments, with $n=4-7$ per group, per experiment. (** $p < 0.01$ in SDR vs. NO DC condition)

Figure 2. IFN- α and IFN- γ mRNA expression is increased, and M1 mRNA expression is decreased in the lung of influenza infected mice receiving SDR DCs prior to infection

The apical lobe of each lung was flash frozen and processed for PCR analysis of (A) M1 (* p < 0.01), (B) IFN- α (* p < 0.04), and (C) IFN- γ (* p < 0.05) mRNA expression. Data represent three separate experiments, with $n=4-7$ per group, per experiment. (** p < 0.05 in SDR vs. NO DC condition)

Figure 3. IFN- γ expression secretion by CD8⁺ T cells is increased when antigen is presented by SDR DCs

CD11c⁺ DCs from SDR or HCC mice were purified and co-cultured with purified CD8⁺ T cells from influenza infected mice in a 1:5 ratio. Cultures were stimulated with 10 μ g/ml NP366-74 peptide and supernatants were harvested after 48 hours. Supernatants were analyzed by cytometric bead array, and only IFN- γ protein levels are shown, as differences in all other cytokines did not reach statistical significance. Data represent three separate experiments, with n=4–7 per group, per experiment. (* $p < 0.05$)

Figure 4. The majority of DCs in SDR and HCC mice are of a conventional phenotype
 CD11c⁺ DCs were purified from SDR and HCC using Pan-DC microbeads (Miltenyi Biotec, Auburn, CA) and analyzed by flow cytometry for expression of CD11c, CD11b, and B220. Cells were gated on the monocyte population and analyzed for co-expression of (A) CD11b/CD11c or (B) B220/CD11c indicating a conventional or plasmacytoid phenotype, respectively. The size of the spleen of each animal in the SDR and HCC group was also assessed to confirm splenomegaly, indicative of that there was an SDR effect (C). Data represent three separate experiments, with n=4–7 per group, per experiment. (*p < 0.001)