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Exposure to cigarette smoke impacts myeloid-derived regulatory cell function and exacerbates airway hyper-responsiveness

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Abstract

Cigarette smoking enhances oxidative stress and airway inflammation in asthma, the mechanisms of which are largely unknown. Myeloid-derived regulatory cells (MDRC) are free radical producing immature myeloid cells with immunoregulatory properties which have recently been demonstrated as critical regulators of allergic airway inflammation. NO (nitric oxide)-producing immunosuppressive MDRC suppress T cell proliferation and airway-hyper responsiveness (AHR), while the $O_2^{\bullet-}$ (superoxide)-producing MDRC are proinflammatory. We hypothesized that cigarette smoke (CS) exposure may impact MDRC function and contribute to exacerbations in asthma. Exposure of bone marrow (BM) derived NO-producing MDRC to CS reduced the production of NO and its metabolites and inhibited their potential to suppress T cell proliferation. Production of immunoregulatory cytokine IL-10 was significantly inhibited, while proinflammatory cytokines IL-6, IL-1β, TNF-α and IL-33 were enhanced in CS exposed BMMDRC. Additionally, CS exposure increased NF-κB activation and induced BM-MDRC-mediated production of $O_2^{\bullet-}$, via NF-κB dependent pathway. Intratracheal transfer of smoke exposed MDRC producing proinflammatory cytokines increased NF-κB activation, reactive oxygen species and mucin production *in vivo* and exacerbated AHR in C57BL/6 mice, mice deficient in Type I IFNR and MyD88, both with reduced numbers of endogenous MDRC. Thus, CS exposure modulates MDRC function and contributes to asthma exacerbation and identifies MDRC as potential targets for asthma therapy.

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INTRODUCTION

Allergen-stimulated dysregulation of immune responses causes airway inflammation in asthmatics [1, 2]. Infiltrating innate immune cells have been implicated as primary contributors of oxidative stress during asthma, while $CD4^+$ T helper cells drive the persistence and resolution of the inflammatory response [3-9]. Free radical species are important mediators of allergic airway inflammation [10-12]. Cigarette smoking enhances the severity of asthma by exacerbating inflammation, oxidative stress and tissue injury in the respiratory tract [13-16]. Cigarette smoke (CS) also has the potential to modulate free radical concentrations in the human airways and regulate the recruitment of inflammatory immune cells [17-21]. In murine models of asthma, exposure to CS has an adjuvant effect on eosinophils and Th2 cytokines [13, 14], and has recently been shown to induce production of IL-1 family proinflammatory cytokine IL-33 which promotes airway inflammation [22-28]. CS exposure also causes activation and recruitment of alveolar macrophages [17-19] and/or other inflammatory cells, and causes production of reactive oxygen species (ROS), all of which contribute to lung inflammation [10, 12, 21]. In humans, exposure to environmental smoke and CS reduces exhaled NO, suggesting a direct effect of CS on NO production in the human airways [20, 21, 29, 30].

We and others have reported that two distinct subsets of immature myeloid cells that generate NO (nitric oxide) and superoxide ($O_2^{\bullet-}$) termed myeloid-derived regulatory cells (MDRC) are important regulators of allergic airway inflammation [31, 32]. NO-producing MDRC suppress, while $O_2^{\bullet-}$-producing MDRC are pro-inflammatory in both *in vitro* T cell responses and *in vivo* airway hyper-responsiveness (AHR) in murine models of asthma. We investigated here whether CS exposure modulated the immunosuppressive potential of MDRC by switching the free radical profile of MDRC. Herein, we report that exposure to CS exposure inhibits MDRC-mediated suppression of T cell proliferation by reducing their production of NO, immunoregulatory cytokines TGF-β and IL-10, while enhancing the production of proinflammatory cytokines, importantly IL-33. Furthermore, exposure to CS switches the phenotype of MDRC to ROS-producing cells via an NF-KB dependent mechanism. Importantly, intratracheal adoptive transfer of smoke exposed MDRC exacerbated ovalbumin induced murine asthma.

MATERIALS AND METHODS

Mice

C57BL/ 6 were obtained from The Jackson Laboratory (Bar Harbor, ME). OT-II mice were provided by Paul Allen (Washington University, St Louis, MO). The original MyD88 deficient mice were obtained under a Materials Transfer Agreement from Dr. Shizuo Akira (Osaka University, Japan) and were generously provided to us by Suzanne M. Michalek (University of Alabama, Birmingham, AL). The IFNAR deficient mice were derived by John Mountz in C57BL/6 background and were provided by Chander Raman (both from the University of Alabama, Birmingham, AL). Mice 6-8 weeks of age were housed under pathogen free conditions in micro-isolator cages and experiments were approved by the institutional animal care and use committee of the University of Alabama at Birmingham.

In vitro differentiation of Bone marrow-MDRC

Bone marrow (BM) cells were flushed from femurs using PBS and were cultured in RPMI medium supplemented with 10% heat inactivated fetal bovine serum, 100U/ml of penicillin and 100ug/ml of streptomycin sulfate, 1mM sodium pyruvate (all cell culture reagents were obtained from Life Technologies, Grand Island, NY) and 50μM 2-mercaptoethanol (Sigma, St.Louis, MI) and containing 20ng/ml Granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN) and 1μg/ml Lipopolysaccharide (LPS from *Escherichia coli*, strain O26:B6, Sigma, MI) as described before [31]. BM cells were cultured for 5 days and non-adherent cells were collected and restimulated for additional 3 days at 37°C.

Flow Cytometry

The *in vitro* differentiated BM-MDRC were prepared for flow cytometry by first incubating in FACS staining buffer (PBS + 3% FBS) containing 2.0 μg/ml of the mAb 2.4G2 (BD Pharmingen, Franklin Lakes, NJ) at 4°C for 30 minutes. These cells were then stained with several fluorochrome conjugated anti-mouse monoclonal antibodies for cellular phenotyping as follows: PE- labeled anti-mouse Gr-1 (clone: RB6-8C5), PerCPcy5.5-labeled anti-mouse Ly-6C (clone: HK1.4), FITC-labeled anti-mouse Ly-6G (clone: 1A8), APC-labeled anti-mouse F4/80 (clone: BM8), PE- labeled anti-mouse PDL-1 (clone: M1H5), PECy-7-labeled anti-mouse CD11c (clone: N418), PE-labeled anti-mouse CD115 (clone: AFS98) (all these antibodies were obtained from eBiosciences, San Diego CA); APCCy-7 labeled anti-mouse CD11b (clone: M1/70) and PE-labeled anti-mouse CD124 (clone: mIL4-R-M1) from BD Biosciences, San Jose, CA; and PE-labeled anti-mouse iNOS (Santa Cruz Biotechnologies, Dallas, TX).

Cigarette Smoke exposure of BM-MDRC

BM-MDRC were exposed to smoke generated from one 3R4F Kentucky Reference Cigarette (University of Kentucky). Cigarette smoke exposure was performed using a TE-10 smoking machine connected to an exposure chamber (Teague Enterprises). Each smoldering cigarette was puffed for 2 s once every minute for a total of 8 puffs at a flow rate of 1.05 l/min to provide a standard puff of 35 cm^3. The total suspended particulate level was 28.1 ± 0.5 mg/m^3 estimated from 2 separate exposures. MDRC were washed twice in PBS after smoke exposure and viability was determined by trypan blue exclusion and cultured for additional 24 hours in RPMI media as described above. Culture supernatants were harvested for cytokine analyses and cell lysates were prepared for western blot analyses as described below.

T cell proliferation

Naïve $CD4^+CD25^-CD62L^{hi}CD44^{lo}$ OVA-specific $CD4^+$T cells were sorted from spleens of OT-II mice and were labeled with CFSE as described before [32]. Co-cultures were carried out using 10^5 T cells and 10^5 smoke exposed or unexposed MDRC and 10^4 BMDC pulsed with OVA peptide. T cell proliferation was measured as dilution of CFSE using flow cytometry after 72h of co-culture.

Western Blot Analyses

Total homogenates were prepared from smoke exposed or control BMMDRC or lung tissue of adoptive transfer recipient mice following i.t. transfer of smoke exposed or control BM-MDRC. Tissues were harvested following perfusion and BAL at day 5 after transfer and 3 days after OVA challenge in WT mice. Samples were homogenized in RIPA buffer containing protease inhibitors (50 μg total protein) were electrophoresed on a 4-20% gradient SDS gels. Transferred PVDF membranes were probed with NF-κB pathway sampler kit for BM-MDRC lysates (Cell Signaling Technology, Inc, Beverly, MA) following manufacturers recommended dilutions. IL-33 expression in BM-MDRC lysates and lung tissue homogenates were detected using anti-IL-33 antibody (Clone 4E9) (Abcam, Cambridge, MA). Levels of β-actin , the loading control was detected using anti-actin antibody (Sigma, MO).

Analysis of cytokines, IgE and Mucins

Cytokine analysis of IL-6, TNF-α, IL-1β, IL-10 and IL-33 was performed on MDRC cell culture supernatants using standard ELISA kits following manufacturer's recommendations (R&D systems, Minneapolis, MN and MyBioSource, Inc., San Diego, CA for IL-33). OVA specific IgE was measured in the first aliquot of the BAL using ELISA following manufacturer's recommendations (BioLegend, San Diego, CA). The levels of Muc5Ac was quantitated in the first aliquot of the BAL using ELISA following manufacturer's recommendations (MyBioSource, Inc., San Diego, CA).

Measurements of Nitric Oxide

Nitric oxide and its metabolites were measured in BM-MDRC cell culture supernatants by Griess Assay (Cayman Chemicals, Ann Arbor, MI) which uses nitrate reductase and converts nitrate to nitrite and measures total NO production [32]. Quantitation of lung cells with potential to produce NO were determined by first staining collagenase extracted lung cells with the fluorescent indicator for NO, DAF-FM-DA followed by flow cytometry as described before [32].

Measurements of superoxide

$O_2^{\bullet -}$ producing MDRC were detected by flow cytometry after staining with myeloid cell-specific antibodies and incubation for 20 min at RT with dihydroxyethidium (DHE, 10 μM; Molecular Probes, Eugene, OR) as described before [32]. The specificity of DHE for $O_2^{\bullet -}$ was validated by inducing a respiratory burst in the sorted myeloid cells by incubation at 37°C for 15 min with phorbol myristate acetate (PMA, 1 μg/ml) or PMA + the NADPH oxidase inhibitor diphenylene iodonium (DPI, 1 μM; Tocris Bioscience, Bristol, UK) , in presence of superoxide dismutase (256mU/ml, Sigma, St. Louis, MO) or in presence or absence of 10μM Pyrrolidine dithiocarbamate (Calbiochem, La Jolla, CA) or in the presence or absence of 5 μM NF- κB activation inhibitor II, JSH-23 (Santa Cruz Biotechnology, Santa Cruz, CA).

Experimental allergic airway inflammation and intratracheal adoptive transfer of MDRC

Mice were sensitized by intraperitoneal injection on d0 and d7 with 50 μg of alum-precipitated OVA (Grade VII, Sigma Chemical, St Louis, MO; < 1 ng lipopolysaccharide per mg) as previously described[32]. On d12, 10^5 smoke exposed MDRC, unexposed MDRC or PBS were transferred intratracheally as described before [32]. On d14, under anesthesia with isoflurane (Schering-Plough Animal Health, Union, NJ), mice were challenged i.n. with 0.03 ml 0.03 % OVA in PBS or PBS alone. At 3 days after challenge, bronchoalveolar lavage fluid (BAL) was collected for Muc5Ac, IL-33 and IgE analysis by ELISA. Lung tissue was homogenized for western blot analyses, inflated and frozen (subset of animals from each experiment), extracted with collagenase as described before [32] for further analyses.

Quantitation of Th1/Th2 lymphocytes in lung tissue

Immune cells were isolated as described before [32]. Briefly, contamination of isolated lung cells with blood was reduced by perfusion of the pulmonary circulation with PBS via the right ventricle following euthanasia and thoracotomy. Airway lavage was performed three times with 0.8 ml of PBS each. A 100 μl aliquot from the first collection of the return lavage fluid, after depletion of cells by centrifugation, was used for the determination of levels of cytokines. The three aliquots of lavage from each animal were pooled and used for determination of total cell numbers and for analysis of myeloid cell subsets. The total number of viable lavage cells was determined using Trypan blue and a hemacytometer. Infiltrating leukocytes were isolated from minced lung tissue by treatment with collagenase-B (2 mg/ml, Roche) and DNase I (0.02 mg/ml, Sigma Chemical) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 10 μg/ml penicillin-streptomycin, 25 μM 2-mercaptoethanol and 0.1 mM non-essential amino acids (Life Technologies) at 37°C for 30 min. This was followed by the addition of an equal volume of IMDM containing 20% FBS. Cell suspensions were filtered using 40-μm cell strainer, washed with PBS, treated with ACK lysis buffer (Quality Biologicals Inc., Gaithersburg, MD) and then pretreated, for 20 minutes, in FACS staining buffer (PBS + 3% FBS) containing 2.0 μg/ml of the mAb 2.4G2 (BD Pharmingen, Franklin Lakes, NJ) at 4°C. These cells were then stained to identify and characterize immune cell populations using cell surface marker specific fluorescence conjugated APC-Cy7-labeled anti-CD3(Clone 17A2)and PECy-7-labeled anti-CD4 (Clone GK1.5) antibodies for 30 min at 4°C (eBioscience, San Diego, CA). For detecting the frequency of IFN-γ, and IL-4 secreting cells, cells (10^6/ml) were cultured in RPMI-1640 containing 5 ng/mlPMA and 500 ng/ml Ionomycin along with 10 μg/ml GolgiPlug protein transport inhibitor (BD Biosciences, Sparks, MD), at 37°C, for 3 hours. Cells were then harvested, fixed and permeabilized using BD cytofix/cytoperm fixation /permeabilization kit (BD Biosciences) and then stained with fluorescently labeled antibodies. Cells were washed twice with PBS before analysis. Flow cytometry acquisitions and analyses were carried out using Becton Dickinson LSR II with FACS Diva software (BD Biosciences, San Jose, CA). Data were further analyzed using FlowJo 10 (Tree Star, Ashland, OR).

Measurement of AHR

Airway resistance was measured in response to increasing concentrations of inhaled aerosolized methacholine (0-50 mg/ml in water) [32]. Mice were anesthetized with ketamine (10 mg/kg intraperitoneal) followed by pancuronium bromide (2.0 mg/kg). After insertion of an 18G tracheostomy catheter, the mice were mechanically ventilated at 160 breaths/ min with a tidal volume of 0.2 ml, and a positive end-expiratory pressure of 2– 4 cm H_2O using a flexiVent apparatus (SCIREQ, Montreal, Canada). Tissue resistance was computed continuously over the period of 20 s to 6 min and an average of 12 readings at 30 s intervals was used for calculations.

Immunofluorescence Microscopy

Tissue sections (6-8 μm) from frozen inflated lung tissue were first fixed in pre-chilled methanol for 10 minutes, washed with PBS followed by incubation for 1 hour at room temperature in 1% BSA in PBS. Tissue sections were then stained with unconjugated anti-NF-κB p65 antibody (Abcam, Cambridge, MA) (diluted 1:200 in 1% BSA in PBS), or anti-CD33 antibody () for 60 minutes, at room temperature. After PBS washes, sections were incubated with Alexa594 or Alexa 488 conjugated goat anti-rabbit secondary antibody, washed in PBS and stained with DAPI. Digital photomicrographs were acquired using an Olympus BX 60 system and processed with Adobe Photoshop software.

Statistical analysis

Data are presented as the mean ± SD. Statistical analysis was performed using Graph Pad Prizm5. One way ANOVA with Tukey multiple comparison post-test and the Student's *t*-test was used for comparisons. Statistical significance was determined at the < 0.05 level.

RESULTS

Characterization of MDRC derived from murine bone marrow precursors

In vitro culture of murine bone marrow-derived cells with GM-CSF and LPS yielded a distinct population of differentiated Gr-1$^+$CD11b$^+$ cells, of which 99% were Ly-6C$^+$ and 94% Ly-6C$^+$ F4/80$^+$ (Fig. 1A) as described before [31]. Further analyses of surface and functional phenotype within these cells showed not only significant expression of intracellular iNOS, but also showed significant expression of the IL-4 receptor α subunit (IL-4Rα or CD124), which comprises part of the receptor for IL-4 and IL-13, and has been postulated as a potential marker for MDSC[33]. The bone marrow derived-MDRC (BM-MDRC) also expressed macrophage-colony stimulating factor receptor (CD115) and the inhibitory PD-L1 receptor of B7 family [33] (Fig. 1B). These Ly-6C$^+$CD115$^+$CD124$^+$PDL-1$^+$ cells were also Ly-6G$^-$CD11c$^{lo\text{-}neg}$ and resembled the phenotype of myeloid-derived suppressor/regulatory cells described in cancer and in airway inflammation [34-36].

Cigarette smoke exposure inhibits nitric oxide production and induces a proinflammatory phenotype in MDRC

We and others demonstrated previously that Gr-1$^+$CD11b$^+$Ly-6C$^+$F4/80$^+$ MDRC suppress T cell proliferation and AHR [31, 32]. MDRC mediated regulation of allergen induced immune

responses is mediated via iNOS derived NO and immunoregulatory cytokines. Therefore, we first investigated whether cigarette smoke (CS) exposure would alter NO production by MDRC and inhibit their regulatory potential. As shown in Fig. 2A, levels of total nitrite and nitrate were significantly reduced in culture supernatants of BM-MDRC following CS exposure in a time-dependent manner without significant effects on the viability of MDRC (data not shown). Co-culture of CS exposed BM-MDRC with CFSE labeled naïve $CD4^+$ $CD44^{lo}$ OVA specific T cells significantly increased T cell proliferation compared to co-cultures with unsmoked-MDRC in which significant T cell suppressive effect was observed (Fig.2B). We then determined the impact of CS exposure on the cytokine profile of BM-MDRC by measuring cytokine levels in MDRC culture supernatants following CS exposure. Significant increase in proinflammatory cytokines IL-6, TNF-α, and IL-1β and reduction of immunoregulatory IL-10 was observed (Fig. 2C).

Cigarette smoke exposure activates NF-κB pathway in BM-MDRC

Several studies have focused on NF-κB as a central inflammatory hub that controls inflammatory processes in CS- induced chronic inflammatory lung diseases [37-40]. Signaling pathways involved in oxidative stress and inflammation generally intersect with NF-κB signaling. We investigated whether CS exposure would lead to downstream activation of NF-κB signaling in BM-MDRC and modulate their function. As shown in Fig.3, CS exposure increased the phosphorylation of IKKα/β, IKBα and NF-κB p65 in BM-MDRC, and decreased the expression of IKBα in the BM-MDRC, suggesting that IKBα is targeted for proteosomal degradation and indicating the activation of NF-κB pathway in BM-MDRC.

Exposure to smoke induces MDRC-mediated ROS production by NF-κB dependent mechanism

We investigated whether CS- exposure while reducing NO production by MDRC, induces a proinflammatory response via ROS production. We determined ROS production from MDRC by first quantitating spectrophotometrically the kinetics of reduction of cytochrome c as described before [32, 41, 42]. Smoke exposure induced a 6-fold increase in $O_2^{\bullet -}$ production by MDRC in presence of PMA which was inhibited in presence of $O_2^{\bullet -}$ -quenching superoxide dismutase (SOD) or with a general NADPH oxidase inhibitor (DPI) (Fig. 4A). Interestingly, this smoke induced ROS production was inhibited in presence of an NF-κB inhibitor, pyrrolidine dithiocarbamate **(**PDTC**)** and NF-κB activation inhibitor JSH-23 suggesting a significant role for an NF-κB dependent regulation of ROS production by BM-MDRC. Furthermore, the percentage of DHE^+ (fluorescent indicator for ROS) MDRC quantitated by flow cytometry was increased following smoke exposure, which was then significantly reduced in presence of PDTC or DPI (Fig. 4B).

Smoke exposure-mediated NF-κB induction is persistent in the absence of endogenous MDRC

Recent evidence suggests that TLR4/MyD88 mediated signaling and mechanisms dependent on IL-1β are essential for the LPS-induced generation of MDRC from bone marrow precursors [31, 43]. LPS induced signaling involves two primary pathways; a MyD88 dependent mechanism for the induction of proinflammatory cytokines and a MyD88

independent mechanism which mediates expression of IFN-inducible genes [44, 45]. Consistent with these previous studies, a significant reduction in LPS and GM-CSF-mediated generation of BMMDRC was observed in mice deficient in MyD88, IFN-AR and IL-1β, (Fig. 4C). Intratracheal transfer of smoked BM-MDRC into MyD88 and IFN-AR deficient mice with reduced endogenous MDRC showed NF-κB activation in the airway (Fig. 4D) compared to transfer recipients of unsmoked MDRC. Taken together, these data suggested prolonged induction of NF-κB pathway in CS- exposed MDRC.

Adoptive transfer of smoke exposed MDRC enhances allergic airway inflammation

Intratracheal adoptive transfer of smoked MDRC into ovalbumin sensitized and challenged WT mice increased the percentage of ROS^+ cells in the lung tissue and reduced the percentage of NO-producing lung cells (Fig. 5A). Total number of infiltrating total $CD4^+$ T cell percentages were also increased following adoptive transfer of smoked MDRC into mice with asthma. Percentages of both Th1 and Th2 lymphocytes were significantly increased, while percentages of $CD4^+IL\text{-}17^+$ remained unchanged in the adoptive transfer recipients of smoked MDRC compared to control MDRC (Fig. 5B). Intratracheal transfer of unsmoked MDRCs into ovalbumin sensitized and challenged mice significantly inhibited airway Muc5Ac and OVA specific IgE in the BAL (Fig. 5C) compared to PBS transferred OVA challenged controls. The levels of Muc5Ac and OVA-IgE in OVA challenged mice were significantly higher compared to PBS challenged controls. Importantly, intratracheal adoptive transfer of smoke exposed MDRC enhanced Muc5Ac and OVA-IgE in OVA challenged mice, the levels of which were significantly higher than detected in transfer recipients of unsmoked MDRC.

Adoptive transfer of smoke exposed MDRC enhances IL-33 production in airways and lungs of mice with allergic airway inflammation

IL-33 is a pleiotropic cytokine predominantly expressed in lung tissue and can elicit airway inflammation in mice [25]. We tested the hypothesis that IL-33 is induced by CS and contribute to CS- mediated exacerbation of airway inflammation. We first determined expression in BM-MDRC which was significantly increased after CS-exposure (Fig. 6A). We also observed increased levels of IL-33 in the lung tissues of mice following adoptive transfer of smoked MDRC compared to control MDRC (Fig. 6B). We then compared levels of IL-33 in the BAL of mice sensitized and/or challenged with OVA before and after transfer of smoked or control MDRC. As shown in Fig. 6C, IL-33 levels were increased in BAL of mice with asthma compared to PBS sensitized mice. Adoptive transfer of control MDRC did not alter IL-33 levels in BAL but transfer of smoked MDRC significantly enhanced BAL levels of IL-33. Additionally, we detected an increase in $IL\text{-}33^+$ cells *in vivo* five days after adoptive transfer and three days after antigen challenge in the lung tissue of adoptive transfer recipients of smoked MDRC compared to control MDRC recipient mice (Fig. 6D).

Adoptive transfer of smoke exposed MDRC exacerbates airway hyper-responsiveness

We then investigated the impact of smoke exposure on the potential of MDRC to regulate AHR. As shown in Fig. 7, WT mice induced with asthma and which were recipients of

smoked MDRC had significantly higher airway resistance compared to recipients of unsmoked MDRC and PBS. We then determined AHR in both IFN-AR and MyD88 deficient mice following intratracheal adoptive transfer of smoked and unsmoked MDRC and compared to controls within these groups of mice. Although a robust dose dependent response to methacholine challenge was not observed in both MyD88 and IFN-AR deficient mice, a significant increase in AHR was noted following transfer of smoked MDRC compared to unsmoked and PBS controls. Thus these studies suggest that smoke exposure switches MDRC from an anti-inflammatory to a significantly proinflammatory functional phenotype which produces ROS via an NF-κB-dependent mechanism and exacerbates allergic airway inflammation.

DISCUSSION

In this study, we provide evidence that exposure to CS has a significant impact on MDRC-mediated regulation of allergic airway inflammation. Cigarette smoke exposed MDRC showed reduced activation of iNOS resulting in reduction of metabolites of NO. Additionally, the enhancement of a proinflammatory cytokine signature including IL-1 family member IL-33 and TNF-α, and inhibition of IL-10 mediated effects was induced upon exposure to CS *in vitro*. Importantly, CS altered the immunesuppressive MDRC, and switched them to a NADPH oxidase mediated- ROS producing proinflammatory functional phenotype by persistent activation of NF-κB dependent mechanism which contributed to exacerbation of AHR and increase in overall allergic airway inflammation.

Ample evidence supports an oxidant- antioxidant imbalance in asthma. Over abundant of oxidants from environmental contributions and antigen exposure burdens our antioxidant defense systems causing this imbalance. Anti-oxidant enzymatic mechanisms including superoxide dismutases (SOD) are reduced in asthmatics aggravating this oxidant-antioxidant imbalance. Exposure to CS enhances this oxidative stress associated with asthma. CS directly generates oxidants and activates cellular sources to generate more oxidants to further aggravate this oxidant-antioxidant balance. Our observation that CS alters the ROS production by MDRC and induces the phenotypic switch to a proinflammatory MDRC is consistent with the impact that CS and oxidants can exert on myeloid-cell lineages. Although direct effects of CS on innate immune mechanisms that regulate airway inflammation have not been well characterized, recent evidence suggest that CS may primarily trigger IL-33 production that stimulates the synthesis of further key proinflammatory cytokines, chemokines and other inflammatory mediators in the airway [22, 23]. Our data presented here clearly suggests a role for CS in impacting molecular mediators, and downstream signaling cascades of innate immune pathways of MDRC including IL-33 and NF-κB signaling to regulate allergic airway inflammation and AHR. IL-33 participates in immuneregulation and inflammation by signaling through Toll like receptor pathways involving My-D88, leading to activation of NF-κB which then drives the transcription of proinflammatory genes that activate enzymatic pathways leading to oxidative stress [24, 46-48]. Our observations with IL-33 induction in smoke exposed MDRC both *in vitro* and *in vivo* are consistent with the potential of CS in inducing activation of down-stream signaling cascades via IL-33.

MDRC subsets have been appreciated as critical regulators of airway inflammation. Recent studies have highlighted the significance of plasticity of the different subsets that drive their differential functions/molecular mechanisms to regulate dysregulated immune responses. Altered inflammatory responses of alveolar macrophages (AM) to cigarette smoking have been implicated in the pathological changes leading to chronic lung diseases [49, 50]. Deactivation of steady-state polarization of AMs from M1 phenotype to an unusual M2-like polarization has been reported in healthy smokers[51]. This reprogramming of macrophage polarization was even more progressive in subjects with chronic obstructive pulmonary disease, with a progressive deactivation of M1 gene expression and progressive induction of M2 phenotype that was quite distinct from healthy smokers[51]. Exposure studies investigating macrophage activation by ambient air pollution particulates including superparamagnetic iron oxide showed suppression of IL-10 production and enhanced TNFα production with an impaired ability to make M1-M2 phenotype transitions[52]. The CS induced plasticity in MDRC function that we are reporting here are thus consistent with the environmental pollution-mediated transcriptional reprogramming of macrophage function reported in these studies. Our studies suggest that CS and its components have a direct effect on the molecular mediators and functional plasticity of MDRC. Our studies also demonstrate a direct inhibitory effect of CS on the immune suppressive mechanisms of MDRC. ROS production that ensued from either the CS-induced oxidant-antioxidant imbalance or activation of NFκB pathway dictated the phenotypic plasticity/maturation of MDRC rendering them proinflammatory and providing them the potential to enhance T cell driven inflammation as well as airway responses. This proinflammatory switch of MDRC function was inhibited by blocking downstream NF-κB signaling cascades suggesting once again the significance of redox-mediated signaling in regulation of immune mechanisms.

We demonstrate here that CS enhances MDRC-mediated production of IL-33. IL-33 has long been known to signal via the IL-1receptor related protein ST-2 and to induce Th2 responses[24]. IL-33 is also a transcriptional regulator and activator of NFκB pathway [47]. Our data suggests that CS-exposure promotes IL-33 production by MDRC and induces ROS production via NFκB pathway and leads to Th2 responses. Our studies, however, do not delineate whether smoke exposure-mediated IL-33 induction and downstream activation of NF-κB leads to induction of proinflammatory genes that activates ROS and drives further IL-33 production in a feed-back mechanism. It remains to be determined whether CS exposure and IL-33-mediated mechanisms would inhibit the potential of MDRC in inducing differentiation/recruitment of regulatory T cells *in vivo*.

Our studies also indicate a role for CS- mediated modulation of these signaling cascades in driving the uncontrolled proliferative response of T cells and airway exacerbations. Although cigarette smoking causes a systemic inflammatory response, inhibition of T cell-mediated responses that drive lung diseases are consistent with exposure to high doses of CS with reductions in Th2 cell infiltration, Th2 cytokine production and reduced eosinophilia[53]. Low- dose exposure does not promote this suppressive response. Although transition from the oxidative burst response phase of the M1 phenotype to the sensitization phase favoring the M2 phenotype occurs also with chronic exposure to smoke, loss of T cell function and the associated reduction in Th2 cytokine production may impact the M2 transition. This may

result in the progressive induction of IgE-producing Th2 response with M2 phenotype but with suppression of atopic inflammatory response. Our studies are more in line with a low dose chronic CS exposure or acute CS exposure rather than a chronic elevated dose CS exposure in which asthmatic response is suppressed despite sensitization. Although MDRC subsets exhibit overlapping M1and M2 features, our data suggest that CS exposure-induces polarization of MDRC resembling M1 to M2 transitions. Future studies are warranted to address a potential dose-dependent CS-mediated induction of plasticity in MDRC. Our studies thus identify new molecular pathways to target MDRC function and MDRC-mediated regulation of airway inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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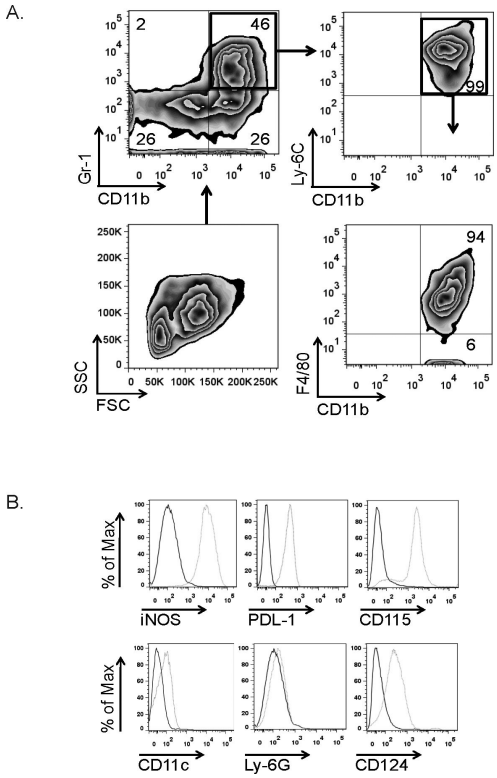

Figure 1. ***In vitro*** **differentiation and characterization of Ly-6C$^+$MDRC from bone marrow precursors**

(A) Flow cytometry plots of differentiated BM-MDRC showing forward and side scatter plots and percentage of Ly-6C$^+$ and F4/80$^+$ cells within the Gr-1$^+$CD11b$^+$ gated cells **(B)** Overlaid histogram flow cytometry plots showing levels of expression of cell surface and intracellular markers that define the phenotype of MDRC. Black lines represent isotype controls and gray lines represent cells positive for the indicated marker.

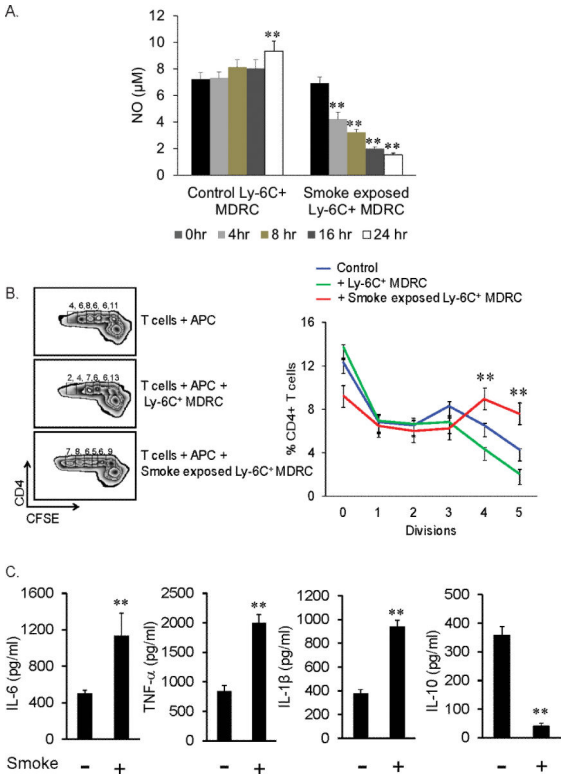

Figure 2. Cigarette smoke exposure inhibits NO production, enhances T cell proliferation and induces a proinflammatory cytokine signature in MDRC

(A) *In vitro* differentiated Ly-6C$^+$ MDRC were immunosorted and were exposed to cigarette smoke as described in methods. Cell culture supernatants were collected at 0-24 hours after culture. Levels of nitrite and nitrate in the culture supernatants were measured in triplicates using Griess assay. Data are Mean ± S.D., **p<0.001 from statistical comparisons of each time point to unexposed controls using ANOVA **(B)** Flow cytometry plots of CFSE labeled 10^5 OVA transgenic T cells co-cultured with 10^5 CS exposed or unexposed MDRC with 10^4 BMDC pulsed with OVA peptide. CFSE dilution was assessed at 72 hours after culture. Right panel shows flow cytometry analyses of CFSE dilution from triplicate experiments of co-cultures similar to that described in (B) was carried out for n=3 samples /group/ experiment. Data are Mean ± S.D., **p<0.001 in comparison of co-cultures with unsmoked MDRC versus smoked MDRC. **(C)** Protein levels of IL-6, TNF-α, IL-1β and IL-10 in cell culture supernatants collected at 24 hours of culture from smoke exposed and unexposed MDRC were determined by ELISA. Measurements were made from triplicate wells for each condition. Data are Mean ± S.D., representative of three independent experiments. **p<0.001 from comparisons of unexposed versus exposed to CS.

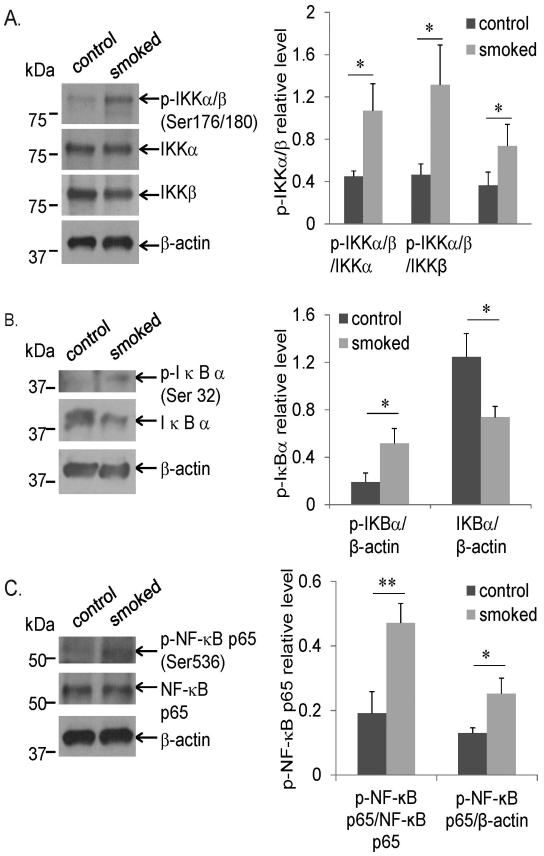

Figure 3. Cigarette smoke exposure activates NF-κB pathway in BM-MDRC

(A-C) Bone marrow cells were cultured in the presence of GM-SCF (10 ng/ml) and LPS (1 μg/ml) for 7 days. Immunosorted MDRC cells were exposed to cigarette smoke as described in methods. Western blot analyses was carried out and relative expression of p-IKKα/β versus IKKα, IKKβ or β-actin was calculated by densitometry and quantified with ImageJ software; relative expression of p-IKBα versus β-actin and IKBα versus β-actin was evaluated and relative expression of p-NF-κB p65 was normalized with NF-κB p65 or β-actin. Data are presented as mean ± SEM of triplicates. * $p < 0.05$, ** $p < 0.01$.

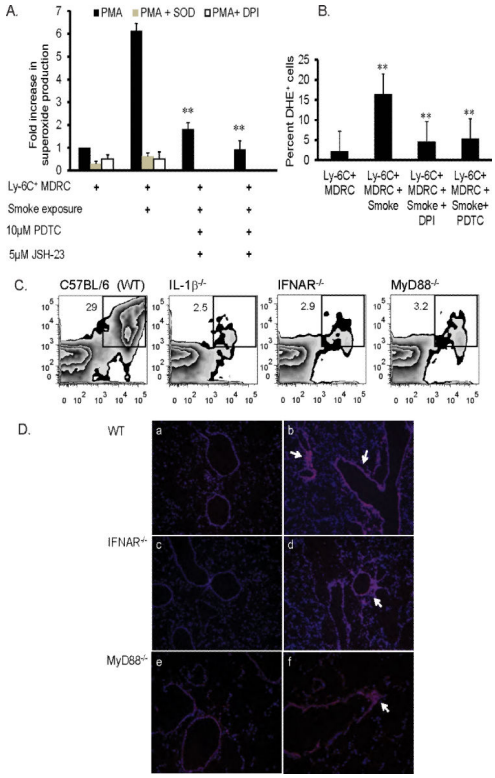

Figure 4. Cigarette smoke exposure increases superoxide production in MDRC via NF-κB dependent mechanism

(A) Fold change in superoxide production was determined by spectrophotometric quantitation and monitoring of kinetics of reduction cytochrome c as described before. **p<0.001 from statistical comparisons of smoked MDRC compared to unsmoked MDRC activated by PMA in presence or absence of PDTC (NF-κB inhibitor), in presence or absence of JSH-23, an inhibitor of transcriptional activation of NF-κB or with SOD **(B)** Percentage of cells with ROS producing potential determined by flow cytometry analyses after staining with DHE, a fluorescent indicator for ROS **(C)** FACS plots showing percentages of *in vitro* differentiated BM-MDRC from WT, IL-1$\beta^{-/-}$, IFNAR$^{-/-}$, MyD88$^{-/-}$ mice **(D)** Immunofluorescence analysis of lung sections showing NF-κB expression in WT recipients of adoptively transferred (a) unsmoked MDRC (b) smoked MDRC (c) IFNAR$^{-/-}$ recipients of unsmoked MDRC (d) IFNAR$^{-/-}$ recipients of smoked MDRC (e) MyD88$^{-/-}$ recipients of unsmoked MDRC (f) MyD88$^{-/-}$ recipients of smoked MDRC

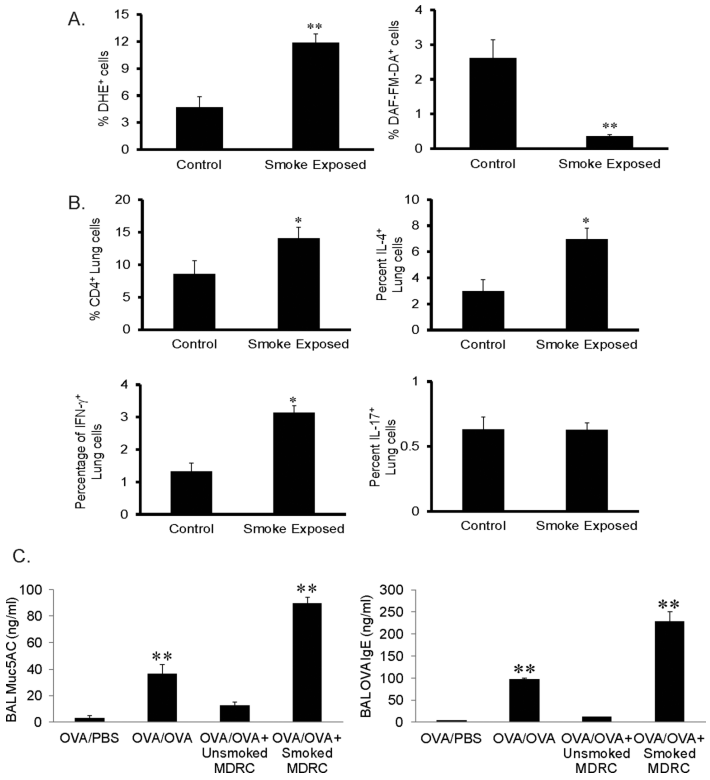

Figure 5. Intratracheal adoptive transfer of smoke exposed MDRC enhanced Th2 responses, BAL IgE and mucin levels *in vivo*

(A) Percentages of lung cells producing ROS and NO were determined from collagenase extracted immune infiltrates in lung tissue following staining with fluorescent indicators, DHE for ROS and DAF-FM-DA for NO and flow cytometry analyses. Lung tissues were harvested from mice with asthma which were adoptive transfer recipients of either control MDRC or smoked MDRC. **p<0.001 from statistical comparisons of adoptive transfer recipients of control versus smoked MDRC. **(B)** Percentages of total $CD4^+$ T lymphocytes and subsets of Th2 ($IL\text{-}4^+$), Th1 ($IFN\text{-}\gamma^+$) and Th17 ($IL\text{-}17^+$) in the lung tissues of mice described in **(A)** determined by flow cytometry analyses. **p<0.001 from statistical comparisons of adoptive transfer recipients of control versus smoked MDRC. **(C- Left Panel)** Levels of Muc5Ac in BAL determined by ELISA. Data are Mean ± S.D. from n=3 mice/group, versus OVA/OVA, OVA/OVA + smoke exposed MDRC compared to both OVA/OVA + unsmoked MDRC and OVA/OVA groups using ANOVA. **(C-Right Panel)** Levels of OVA IgE in BAL determined by ELISA. Data are Mean ± S.D. from n=3 mice/ group, **p<0.001 from statistical comparisons of OVA/PBS versus OVA/OVA, OVA/OVA + smoke exposed MDRC compared to both OVA/OVA + unsmoked MDRC and OVA/OVA groups using ANOVA

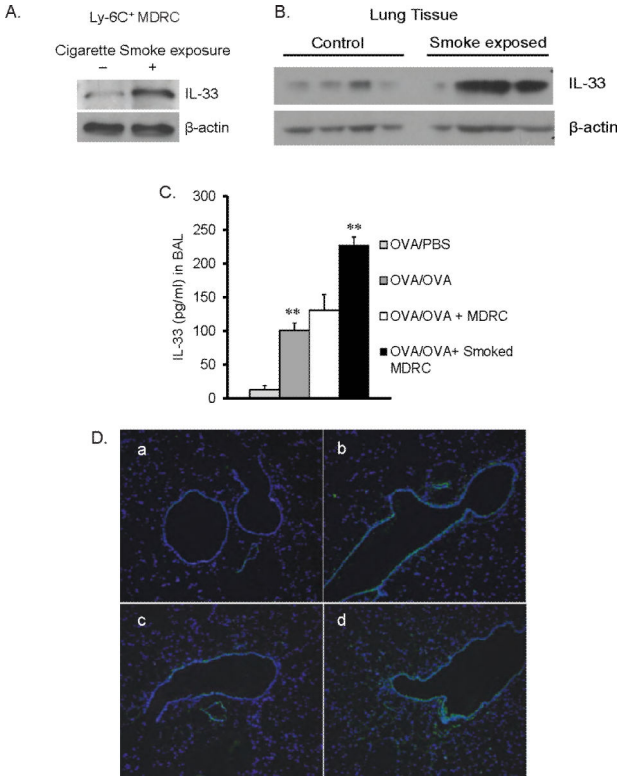

Figure 6. Smoke exposure promotes IL-33 production in MDRC in vitro and *in vivo*

(A) Western blot of control and smoke exposed BM-MDRC probed with anti-IL-33 and anti-β actin antibodies showing increased IL-33 expression after CS exposure. **(B)** Western blot analyses of perfused lung tissue samples of ovalbumin sensitized wild type C57BL/6 mice harvested at day 5 after intratracheal transfer of control and smoke exposed BM-MDRC and 3 days after intranasal ovalbumin challenge probed with anti-IL-33 and anti-β actin antibodies showing increased IL-33 expression after CS exposure. **(C)** Levels of IL-33 detected by ELISA in BAL fluid harvested at 5 days after adoptive transfer and 3 days after ovalbumin or PBS challenge from adoptive transfer recipients of unsmoked and smoked MDRC compared to controls. ** $p < 0.01$ compared to controls. **(D)** Immunofluorescence analysis of lung sections harvested at 5 days after adoptive transfer and 3 days after ovalbumin challenge showing IL-33 expression in (a) WT ovalbumin sensitized mice (b) WT ovalbumin sensitized and challenged mice, WT ovalbumin sensitized and challenged mice which are recipients of adoptively transferred (c) unsmoked MDRC (d) smoked MDRC.

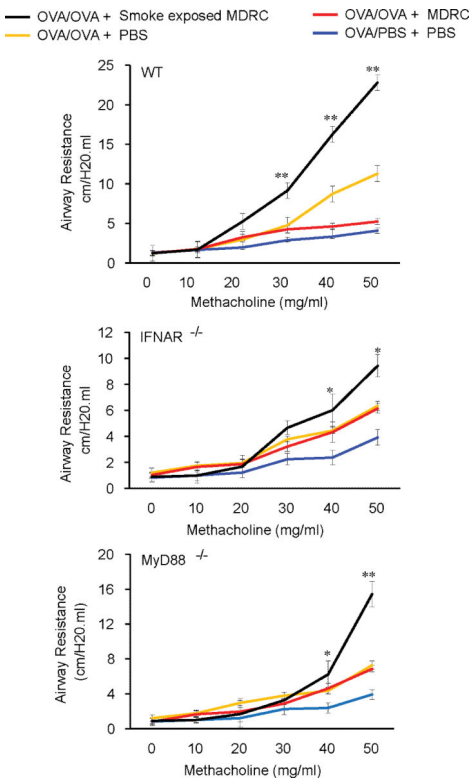

Figure 7. Intratracheal adoptive transfer of CS exposed MDRC exacerbates airway hyper-responsiveness in antigen challenged mice with asthma

Change in airway resistance was measured using flexiVent in OVA sensitized mice at Day3 after intranasal challenge with OVA (5 days after intratracheal transfer of smoke exposed and unexposed MDRC) in n=6-8 animals/group for OVA/PBS (OVA sensitized and PBS challenged mice), OVA/OVA +PBS (OVA sensitized and OVA challenged with intratracheal transfer of PBS), OVA/OVA + MDRC (OVA sensitized and OVA challenged with intratracheal transfer of unsmoked MDRC) and OVA/OVA + smoke exposed MDRC (OVA sensitized and OVA challenged with intratracheal transfer of smoked MDRC). ** $p < 0.01$ in comparison with transfer of unsmoked MDRC and controls, * $p < 0.05$ in comparison with adoptive transfer of unsmoked MDRC and controls.