Reduced immune responses in chimeric mice engrafted with bone marrow cells from mice with airways inflammation

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Short Title: Immune responses in chimeric mice

Abbreviations: ADLN, airway-draining lymph nodes; BALF, bronchoalveolar lavage fluid; BM, bone marrow; DC, dendritic cell; DNBS, dinitrobenzene sulfonic acid sodium salt; DNFB, 2,4-dinitro-1-fluorobenzene; EAAD, experimental allergic airways disease; FITC, fluorescein isothiocyanate; FLT3-L, Fms-like tyrosine kinase 3-Ligand; GM-CSF, granulocyte-macrophage colony stimulating factor; OVA, ovalbumin; PGE_2, prostaglandin E_2; SDLN, skin-draining lymph nodes.

Abstract

Objective During respiratory inflammation, it is generally assumed that dendritic cells differentiating from the bone marrow are immunogenic rather than immunoregulatory. Using chimeric mice, the outcomes of airways inflammation on bone marrow progenitor cells was studied.

Methods Immune responses were analysed in chimeric mice engrafted for >16 weeks with bone marrow cells from mice with experimental allergic airways disease (EAAD).

Results Responses to sensitization and challenge with the allergen causing inflammation in the bone marrow-donor mice were significantly reduced in the chimeric mice engrafted with bone marrow cells from mice with EAAD (EAAD-chimeric). Responses to intranasal LPS and topical fluorescein isothiocyanate (non-specific challenges) were significantly attenuated. Fewer activated dendritic cells from the airways and skin of the EAAD-chimeric mice could be tracked to the draining lymph nodes, and may contribute to the significantly reduced antigen/chemical-induced hypertrophy in the draining nodes, and the reduced immune responses to sensitizing allergens. Dendritic cells differentiating *in vitro* from the bone marrow of >16 week reconstituted EAAD-chimeric mice retained an ability to poorly prime immune responses when transferred into naïve mice.

Conclusions Dendritic cells developing from bone marrow progenitors during airways inflammation are altered such that daughter cells have reduced antigen priming capabilities.

Keywords

Bone marrow
Respiratory inflammation
Chimeric mice
Dendritic cells
Immunoregulatory

Introduction

During inflammation, the production of increased numbers of hematopoietic cells, particularly myeloid cells, at the local site and by the bone marrow (BM) is required [1-6]. The body also has innate homeostatic processes to limit inflammation and ongoing tissue damage. Due to the importance of tissue preservation, evolution dictates the involvement of protective pathways. For dendritic cell (DC) differentiation from BM, the development of cells with less immunogenic and more regulatory, or even tolerant properties would help to curb ongoing inflammation in the periphery [5, 6]. We have published that inflammation in the airways of allergen-sensitized mice programmes DC progenitors in the BM such that they consistently develop into cells that are less immunogenic irrespective of the conditions *in vitro* under which they develop [7]. Further, if injected into pre-sensitized mice, subsequent responses to the sensitizing antigen are reduced [7]. The imprinting by airways inflammation on the BM DC progenitors is by a pathway inhibited by indomethacin, a cyclooxygenase inhibitor. Further, administration of the lipid mediator, prostaglandin E_2 (PGE_2), to mice can stimulate the differentiation in BM of poorly immunogenic DCs [8, 9]. These experiments have involved the culture and expansion of BM cells *in vitro*, with the function of the DCs tested by transfer into new mice. There are multiple pathways by which PGE_2 can regulate immune responses [10]. Furthermore, PGE_2 effects on bone marrow cells have been reported with enhanced hematopoietic stem cell homing, survival and proliferation [11].

The aim of this study was to allow the DC progenitors from the BM of mice with inflammation of the airways to differentiate *in vivo*, not *in vitro* as described above for previous experimentation. In the study reported here, chimeric mice were established with BM cells from mice with inflammatory airways. Thus the need for *in vitro* expansion of DC progenitors from the BM and adoptive transfer experiments was removed. The chimeric mice were allowed to fully engraft with the transferred cells before their immune status was assessed. During this engraftment period (16 weeks), DC progenitors in the BM would have differentiated and seeded the peripheral tissues of the BM-ablated recipient mice under steady state conditions. The responses to immune challenges in the chimeric mice engrafted with BM from mice with experimental allergic airways disease (EAAD), were investigated.

Materials and Methods

Mice and Ethics Statement

Female C57BL/6J (CD45.2 alloantigen) and B6.SJL-Ptprca (CD45.1 alloantigen) mice were obtained from the Animal Resources Centre (Murdoch, Western Australia). All experiments were performed with the approval of

the Telethon Kids Institute Animal Ethics Committee, (approval #235), and standard operating procedures for euthanasia and anesthesia defined according to guidelines of the National Health and Medical Research Council of Australia.

Induction of experimental allergic airways disease

As previously published for development of EAAD [7,9], mice (B6.SJL-Ptprca as donors for chimeric mice or fully engrafted chimeric mice) were injected intraperitoneally with ovalbumin (OVA) (Sigma-Aldrich, St Louis, MO) in alum (aluminium hydroxide suspension, Serva, Heidelberg, Germany) on day 0 (10 μg OVA with 2 mg alum in 200 μl saline per mouse), and again on day 14. These mice were then challenged for 30 min on day 21 with a 1% OVA-in-saline aerosol (UltraNeb, DeVibiss, Somerset, PA). Twenty-four hours later, some mice were euthanased, and the BM harvested for transfer to new mice. Alternatively, mice were further challenged two times with OVA aerosol on days 22 and 23 and 24 hours after the last challenge, the mice were euthanased, and airway draining lymph nodes (ADLN; mediastinal, tracheobrachial, and parathymic) and bronchoalveolar lavage fluid (BALF) collected.

Generating chimeric mice

As previously reported for BM cell isolation [7, 8, 10], tibias and femurs were flushed using a glucose-potassium-sodium buffer containing 10% FCS (SAFC Biosciences, Brooklyn, Australia). Disaggregated BM cells were passed through cotton wool to remove bone debris. Red blood cells were lysed with ammonium chloride. Eight-week old C57BL/6J mice (recipients, CD45.2 alloantigen) were γ-irradiated (2 x 550 rad) using a ^{137}Cs source (Gammacell 3000 Elan, MDS Nordion, Ottawa, Canada) prior to injection of 2 x 10^6 BM cells from congenic B6.SJL-Ptprca (CD45.1 alloantigen) mice with EAAD. Control chimeric mice were γ-irradiated and injected with 2 x 10^6 BM cells from naïve congenic B6.SJL-Ptprca mice. Age matched mice that had not been γ-irradiated were used as a control for chimeric mice (non-chimeric). Mice were provided polymyxin B sulfate (Sigma-Aldrich) and neomycin trisulfate salt hydrate (Sigma-Aldrich) in their drinking water for 2 weeks before, and after γ-irradiation and BM cell reconstitution. As published [7], in the donor BM cells from EAAD mice there was a small but significant increase in myeloid ($Gr1^+CD11b^+$) cells, with a small compensatory decrease in lymphoid ($B220^+CD11b^-$), and erythroid ($Ter119^+CD11b^-$) populations.

The engraftment of cells in the chimeric mice was tracked in the blood, BM, spleen and lymph nodes [12]. Blood was collected into K_2EDTA coated microtainer tubes (BD, Franklin Lakes, NJ) and cell profiles analysed using the Advia 120 hematology system (Siemens Healthcare Diagnostics Inc, Tarrytown, NY). At 2, 3, 4, 8, 12 and 18 weeks post-reconstitution, freshly isolated BM, spleen and lymph node cells were counted, and an aliquot stained with combinations of APC anti-CD45.1, FITC anti-CD45.2, PE anti-CD19, PE-Cy7 anti-B220, PE anti-CD4, PE-Cy7 anti-CD3 and PE anti-CD11c antibodies. To prevent nonspecific antibody binding, cells were pre-incubated for 5 min with anti-CD16/CD32 Fc receptor antibody (BD Pharmingen). Cells were incubated with antibodies for 30 min before washing, and data collection on an LSRII flow cytometer (BD Biosciences). All incubations and cell washes were performed at 4°C. Cells were then analysed for CD45 expression, and classified as $CD4^+CD3^+$ T cells, $CD19^+B220^+$ B cells, or $CD11c^+$ DCs. FlowJo software (Treestar, version 9.5.2, Ashland, OR) was used for flow cytometric analysis.

Culture and phenotyping of cells in the ADLNs

ADLN were harvested, physically disaggregated, and filtered through 100 μm nylon mesh into single-cell suspensions [7, 9]. Cells (10^5 cells/well, 8 replicates per mouse) in RPMI-1640 medium (Hyclone, Thermo Scientific, Waltham, MA) containing 10 % FCS, 2 mM L-glutamine (Sigma Aldrich), 50 μM 2-mercaptoethanol, and 5 μg/ml gentamicin (Sigma Aldrich) (RPMI-10) were cultured at 37 °C with 5% CO_2 with or without 100 μg/ml OVA. Methyl-^{3}H-thymidine (Perkin-Elmer, Boston, MA; 0.25 mCi; 10 μl/well) was added after 72 h and cells harvested at 96 h. Cell proliferation was measured by ^{3}H-thymidine incorporation. Alternatively, ADLN cells were stained for 30 min with FITC anti-CD3, BD Horizon PE-CF594 anti-CD4, BD Horizon V450 anti-CD8, PE anti-CD19, PE anti-B220, APC anti-CD11c, and biotinylated anti-I-A/I-E antibodies. Cells were pre-incubated for 5 min with rat anti-mouse CD16/CD32 Fc receptor antibody to prevent non-specific binding. Where necessary, cells were incubated for a further 30 mins with SA-APC-Cy7. Data were collected on a BD LSRFortessa flow cytometer (BD Biosciences). FlowJo software (Treestar, version 9.5.2, Ashland, OR) was used for flow cytometric analysis.

Bronchoalveolar lavage

BALF was obtained via an incision in the trachea, 500 μl of 0.2% BSA (Sigma-Aldrich) in PBS was flushed into the lungs, recovered, and repeated twice to obtain a 1 ml sample. Lavage samples were centrifuged (300 g, 4°C, 7 min), and the pellet resuspended in 100 μl to count and for preparation of a cytocentrifuge smear.

Measurement of serum OVA-specific IgG1 and IgE

Serum was collected from mice 6 h after the second OVA challenge on Day 22. Wells of a 96-well plate were coated with OVA (1 μg) overnight at 4°C in carbonate buffer (100 mM Na_2CO_3, 100 mM $NaHCO_3$, pH 9.6), washed, incubated with blocking buffer (0.5% BSA in Tris-HCl, pH 7.4) before incubation for 2 h at room temperature with serum samples, diluted serially 10-fold in a Tris-based assay buffer. Biotinylated mouse anti-IgG1 (Southern Biotechnology Associates Inc, Birmingham, Alabama) or biotinylated mouse anti-IgE antibody (Biolegend, San Diego, CA) was added before IgG1 or IgE was measured in a dissociation-enhanced time-resolved fluorescence immunoassay with europium as the label [13].

PKH26 and LPS

Chimeric mice >16 weeks post reconstitution or age-matched non-chimeric controls were given intranasal PKH26 diluted 1 in 100 in diluent C (part of PKH26 kit, Sigma-Aldrich). After 8 h, half the PKH26-exposed mice were given intranasal LPS (10 μg). The mediastinal lymph node was harvested 48 h after PKH26 administration, disaggregated and cells stained for 30 min with BD Horizon V450 anti-CD8α, APC anti-CD11c and FITC anti-I-A/I-E antibodies. Data were collected on a BD LSRFortessa flow cytometer (BD Biosciences).

FITC uptake and cell migration assay

The dorsal skin of chimeric mice >16 weeks post reconstitution or age-matched non-chimeric controls was shaved, and administered 100 μl 0.5% w/v fluorescein isothiocyanate (FITC) (Sigma-Aldrich) prepared in acetone 1:1 dibutylphthalate [12]. After 16 h, the skin-draining lymph nodes (SDLN, axillary, brachial and inguinal) were harvested from mice, physically disaggregated, and stained with APC anti-CD11c, PE anti-I-A/I-E, PE-Cy7 anti-CD8α, biotinylated anti-CD45.2, BD Horizon V450 anti-CD45.1 antibodies and if required, SA-APC-Cy7.

5-Aza-2'-deoxycytidine

5-Aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich), a DNA methylation inhibitor, was used as previously described [10]. 5-Aza-dC was dissolved in DMSO at 5 mg/ml before further dilution in saline. OVA-sensitized and boosted mice were injected intraperitoneally with 5-aza-dC at 0.2 mg/kg (or similarly diluted DMSO) one hour before receiving 1% OVA-in-saline aerosol on day 21. The mice were again injected intraperitoneally with 5-Aza-dC (0.2 mg/kg or diluted DMSO) on days 22 and 23 before bone marrow harvest on day 24.

Adoptive transfer of BM-differentiated $CD11c^+$ cells into the ears of naïve mice and subsequent contact hypersensitivity assay

Freshly-isolated BM cells (from chimeric mice or mice injected with 5-Aza-dC) were cultured in RPMI-10 at a density of 8×10^5 cells/ml in 24 well plates with 10 ng/ml GM-CSF and 10 ng/ml IL-4 (Peprotech Inc, Rocky Hill, NJ) for 6 days to promote $CD11c^+$ cell differentiation (medium replaced on days 2 and 4). Non-adherent cells were enriched to >95% $CD11c^+$ cells (confirmed by flow cytometry) using anti-CD11c magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and autoMACS (Miltenyi Biotec) separation. Enriched $CD11c^+$ cells at 10^6 cells/ml in RPMI-10 were pulsed with 1 mM 2,4-dinitrobenzene sulfonic acid sodium salt (DNBS) (MP Biomedicals LLC, Solon, OH) for 30 min at 37°C before washing, resuspension at 5×10^7 cells/ml in 0.9% saline, and 10^6 cells injected into the ear pinnae of naïve C57BL6/J mice (n = 8 ears of 4 mice per group). After 7 days, each side of the ears was painted with 10 µl 0.2% 2,4-dinitro-1-fluorobenzene (DNFB) (Sigma-Aldrich) in acetone:olive oil (4:1); DNFB is the non-water soluble form of DNBS, but with the same antigenic moiety as DNBS [7, 8, 12]. Ear thicknesses were determined before, and 24 h after challenge with DNFB using a spring-loaded micrometer (Mitutoyo, Aurora, IL). The ear thickness before painting was subtracted from that measured after 24 h.

Statistical Analysis

Statistical analysis was performed using the Student *t* test or one-way ANOVA with bonferroni's correction. Differences were considered statistically significant when $P<0.05$.

Results

Engraftment of the chimeric mice

To generate the chimeric mice, BM was taken from CD45.1 mice, with or without EAAD [7], and injected into γ-irradiated recipient mice. The cell recoveries from the BM, spleen and lymph nodes of the chimeric mice (2-18 weeks post injection) were very similar for all mice, with the cell numbers isolated from tissues similar to that of age-matched, non-chimeric mice after 12 weeks (Fig. 1a). In the spleen and lymph nodes of the chimeric mice, >90% of recovered cells were progeny of donor (CD45.1) BM cells (Fig. 1b). In BM, approximately 50% cells are $Ter119^{+}CD11b^{-}$ erythroid cells [7, 8] (Fig. 1b). The percentage of $CD19^{+}B220^{+}$ B cells, $CD3^{+}CD4^{+}$ T cells and $CD11c^{+}$ DCs was similar in the control-chimeric (-EAAD) and EAAD-chimeric mice, and after 8 weeks these hematopoietic tissues of the chimeric mice had a cellular profile similar to that of age-matched, non-chimeric mice (Fig. 1c-e).

Hematopoietic cells in the blood of the chimeric mice were analysed 16 weeks after engraftment. In comparison with the non-chimeric mice, and for unknown reasons, there were increased circulating white blood cells in the chimeric mice (Table 1). Importantly, there was no significant difference in circulating red or white blood cells between the 2 types of chimeric mice (control-chimeric and EAAD-chimeric).

Immune responses by cells in the airway, skin and bone marrow were examined in the fully engrafted control- and EAAD-chimeric mice (>16 weeks since injection of donor BM cells).

Reduced responses to OVA sensitization and/or challenge in the EAAD-chimeric mice

To investigate the potential transfer of airway-specific effects from donor mice with airway inflammation, the chimeric mice after 16 weeks engraftment were sensitized and boosted with OVA and alum, and 7 days later challenged on 3 consecutive days with aerosolized OVA. After 24 h, there was a significantly reduced hypertrophy in the ADLN of the EAAD-chimeric mice (Fig. 2a). Despite reduced cell numbers recovered from the ADLN of the EAAD-chimeric mice, the proportions of $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cells, $CD19^{+}B220^{+}$ B cells and $CD11c^{+}$ DCs remained the same (Fig. 2b). Also, when equal numbers of non-fractionated cells from

the ADLN of the chimeric mice (+EAAD) were cultured without, or with OVA, their proliferation was very similar (Fig. 2c) suggesting quantitative, not qualitative, changes to cell types in the ADLN. Thus, the data suggest reduced responses to OVA in the nodes accounts for, and is proportional to, reduced hypertrophy of the ADLN. The result may reflect reduced sensitization to OVA or alternatively, an ameliorated response to OVA challenge may have been the primary determinant of the results of Fig. 2a-c.

The yield of cells recovered from the BALF of the EAAD-chimeric mice was significantly reduced compared with those in the BALF of control (-EAAD) chimeric mice (Fig. 2d). There were fewer cells in the BALF of the non-chimeric mice relative to the chimeric mice and may reflect the significantly reduced number of circulating white blood cells in the non-chimeric mice (Table 1). The percentage of eosinophils and neutrophils in the BALF was similar in the +EAAD-chimeric (29.4+7.7 and 4.7+1.7, n=7, mean+SEM), the control-chimeric (-EAAD)(33.2+9.3 and 6.1+1.5) and the non-chimeric mice (36.7+11.3 and 2.3+1.1), respectively (Fig. 2e). Immunological consequences of the reduced responses to OVA in the EAAD-chimeric mice was shown by reduced levels of circulating OVA-specific IgE and IgG1 in these mice when compared to levels measured in the control-chimeric mice (-EAAD)(Fig. 2f, g).

Reduced trafficking to nodes by DCs in the airways of EAAD-chimeric mice

To investigate further the reduced responses to OVA sensitization and/or challenge and to study in more depth the activity of airway DCs, cells in the airways of the chimeric mice, as well as age-matched non-chimeric mice, were labelled with the non-activating dye, PKH26, administered intranasally. Eight h later, LPS was delivered intranasally for activation of airway DCs, stimulation of their trafficking to the draining nodes and initiation of an inflammatory response. In response to LPS, after 40 h, the number of cells in the mediastinal lymph node of non-chimeric and the control-chimeric mice (-EAAD) increased significantly. However, there was no increase in cells in the mediastinal lymph node of LPS-administered EAAD-chimeric mice (Fig. 3a; the number of cells in the mediastinal lymph nodes for the +EAAD-chimeric mice was 26% of those for the control-chimeric mice (-EAAD)). In the node of the EAAD-chimeric mice, there were significantly fewer $CD11c^{+}$I-A/I-E^{+} DCs (Fig. 3b; 32% of those in the control-chimeric mice (-EAAD)), including significantly reduced PKH26-labelled DCs (Fig. 3c; 9% of those in the control-chimeric mice (-EAAD)). In the absence of LPS administration, there were few $PKH26^{+}$ cells in the airway draining lymph nodes. Thus, there is a suggestion that the total number of cells measured in the mediastinal lymph node of LPS-administered mice may be proportional to the number of

incoming DCs (Fig. 3a). As shown graphically in Fig. 3d and 3e for cells from representative chimeric mice (+EAAD), the $PKH26^{+}CD11c^{+}I\text{-}A/I\text{-}E^{+}$ cells were $CD8\alpha^{-}$, and represented migratory DCs, and not lymphoid-resident DCs that had taken up soluble dye [14].

Reduced trafficking to nodes by DCs in the skin of EAAD-chimeric mice

To investigate whether activated DCs from non-respiratory tissues of the EAAD-chimeric mice were poor at trafficking to draining nodes, shaved dorsal skin of these mice were administered the inflammatory hapten, FITC. After 16 h, the cells in the SDLN were counted. In the absence of FITC, there were very similar numbers of cells in the SDLN. FITC stimulated a significant hypertrophy of the SDLN in the control-chimeric mice (-EAAD) and the non-chimeric mice, but not in the EAAD-chimeric mice (Fig. 4a). To further characterize which skin cells had delivered FITC to the draining nodes, the $CD11c^{+}I\text{-}A/I\text{-}E^{+}$ cells in the SDLN (Fig. 4b) were examined for FITC expression. The $CD11c^{+}I\text{-}A/I\text{-}E^{hi}$ cells expressed elevated levels of FITC (solid line of Fig. 4c), were $CD8\alpha^{-}$ (Fig. 4d) and were ~90% CD45.1 suggesting that they were migratory DCs of donor origin and not residual skin cells after γ-irradiation of the mice [14,15](Fig. 4e). The number of $FITC^{+}CD11c^{+}I\text{-}A/I\text{-}E^{hi}$ cells in the SDLN of the EAAD-chimeric mice was 63% of those measured in the control-chimeric mice (-EAAD)(Fig. 4f) and was equivalent in proportion to the total cell numbers in the nodes as shown in Fig. 4a.

Poorly immunogenic dendritic cells differentiate from the bone marrow of EAAD-chimeric mice 16 weeks after engraftment

BM cells of the chimeric mice (16 weeks after engraftment), were harvested for assay of the ability of differentiated DCs to prime immune responses in new mice (Fig. 5a). After culture to stimulate differentiation and expansion, the DCs were DNBS-loaded and transferred into the ears of naïve mice. After 7 days, DNFB was applied topically to the ears and the swelling of the ears measured after 24 h. DCs differentiated from the BM of the control-chimeric mice (-EAAD) initiated a significant response to DNFB whilst those from the BM of the EAAD-chimeric mice induced a poor, much reduced response (Fig.5b).

Removal of the effect of EAAD on bone marrow cells by administration of 5-Aza-dC to mice

OVA-sensitized and -boosted mice were injected with 5-Aza-dC one hour before, and 24 and 48 h following receipt of the 1% OVA-in-saline aerosol. Bone marrow cells were harvested 72 h after OVA aerosol exposure, cultured with GM-CSF + IL-4 for DC differentiation and subsequent analysis of their priming ability *in vivo*. DCs differentiated from the bone marrow of EAAD mice treated with 5-Aza-dC were no longer less competent to prime immune responses than those differentiated from the bone marrow of control mice (-EAAD mice + 5-Aza-dC)(Fig. 6).

Discussion

The analyses of the engraftment of the donor (CD45.1) cells in the chimeric mice suggested that under steady state conditions, there were no deficiencies in the ability of donor BM-derived DCs to seed the peripheral tissues of the recipient chimeric mice (CD45.2). However, upon challenge with antigens/stimuli reliant on DC functional ability, a dramatic phenotype in the EAAD-chimeric mice was detected. In the EAAD-chimeric mice, the airway $CD45.1^+$ DCs responded poorly to LPS, whilst skin $CD45.1^+$ DCs responded inefficiently to the chemical hapten, FITC. In response to aerosolized OVA by the OVA-sensitized EAAD-chimeric mice, there was a significantly reduced hypertrophy of the ADLN resulting in inhibited production of OVA-specific IgG1and IgE, and reduced cellular infiltration into the lungs. In more DC-specific analyses using intranasal PKH26, followed by LPS administration, fewer PKH26-laden DCs were detected in the draining lymph nodes and suggested that the airway DCs were poor at trafficking to the draining nodes. Similarly, at another site, FITC-laden, skin DCs were not migrating to the draining nodes in significant numbers to stimulate lymph node hypertrophy. Of those $PKH26^+$ and $FITC^+$ DCs that reached the draining nodes in the EAAD-chimeric mice, they were phenotypically similar to those in the nodes of the PKH26/LPS- and FITC-administered control-chimeric mice (-EAAD). The results suggest that any increase in the numbers of cells in the draining lymph nodes is proportional to the number of $PKH26^+$ or $FITC^+$ cells migrating to the nodes. It is not clear why some DCs, upon activation, in the EAAD-chimeric mice were unable to efficiently traffic to the nodes. In previous studies, adoptive transfer of hapten-loaded $CD11c^+$ cells differentiated from the BM of mice with alum-induced peritoneal inflammation, or UV-induced skin inflammation, reduced the contact hypersensitivity response in hapten-presensitized recipient mice [7, 16]. Thus, an 'active' process of regulation by $CD11c^+$ cells must occur that could not reflect a loss of viability by DCs differentiated from the BM of mice with tissue inflammation.

In these experiments BM was taken from allergen-sensitized mice 24 h after allergen aerosol challenge and a time of confirmed inflammation in the airways and their draining nodes [7]. Further, at this time, there was increased myelopoiesis in the BM [7]. Non-fractionated BM cells from these mice, as well as from control mice without airways inflammation, were used to generate the chimeric mice; they were injected into γ-irradiated naïve recipients and allowed to seed all hematopoietic tissues over the subsequent 16 weeks. The longevity of the effect of inflammatory airways on DC progenitors in the BM is a matter of considerable interest. It could be argued that the minimal immune responses and lesser activity of peripheral tissue DCs in the EAAD-chimeric mice were a product of changes in the transplanted BM progenitors several weeks before the 16 week post-engraftment assay time. However, experiments were performed with BM itself harvested 16 weeks after engraftment and the immunogenicity of DCs differentiating from their BM examined. In contrast to DCs from control-chimeric mice (-EAAD), DCs from EAAD-chimeric mice were very poor at initiating immune responses in naïve mice and demonstrated the long-lasting effect of EAAD on hematopoietic progenitors *per se*. The studies with 5-Aza-dC, an inhibitor of DNA methyltransferase [17] and, thus, an inhibitor of DNA methylation, suggested that products of airways inflammation may cause methylation-associated changes directly or indirectly (as a secondary event) to cells in the bone marrow. The identity of a hematopoietic progenitor (myeloid or lymphoid) or mesenchymal cell directly imprinted with change due to inflammation in the airways is the subject of ongoing research and may not be initially in the bone marrow. Further, it is not known if daughter cells have similar methylation changes to their parent cells; it is possible that the result of methylation differences is an altered transcriptome in daughter cells. If the effect is still evident in BM cells 16 weeks after injection of marrow-ablated mice with BM cells, this suggests that early hematopoietic progenitors, even hematopoietic stem cells, have been altered directly or indirectly by a methylation-dependent process [11, 18, 19]. This study concentrated on DCs; it is possible that lymphocytes also differed between the control-chimeric (-EAAD) and EAAD-chimeric mice and also contributed to differences in OVA sensitization and/or challenge.

The first responses investigated in the chimeric mice were those to challenge by the same allergen used to create inflammation in the BM-donor mice. However, DC-dependent responses in the airways and skin to non-specific stimuli (LPS and FITC) were also significantly reduced in the EAAD-chimeric mice and suggested that DCs, a cell type important to innate immunity, had retained a characteristic initiated in DC progenitors in the BM of the donor mice. Thus, an effect of airways inflammation on hematopoietic progenitor cells in the BM had occurred such that DCs of reduced immunogenicity had differentiated from that BM and seeded airways and

skin tissue sites. This result supports the emerging concept of a form of 'memory' within innate immune cells [20]. Our finding that EAAD imprints a change in BM myeloid progenitors (possibly indirectly by effects on other cell types) such that the differentiating DCs have poor immunogenicity upon activation, can be likened to the findings of Yanez et al [21]. That group reported that in both mouse and human experimental systems, detection of a TLR2 agonist by hematopoietic stem and progenitor cells impacted the function of the macrophages they produced. It is proposed that airways inflammation impacts non-specifically on the function of the DCs that differentiate from the BM of inflamed mice. This has been called 'training innate immunity' [20].

The BM is the main site of renewal of hematopoietic cells; DC progenitors in the BM are very important for replenishing exhausted and dying cells in the inflammatory airways of asthma patients [22-25]. This study suggests that inflammatory processes in the airways induce the differentiation of poorly immunogenic DCs from the BM; these cells will, in turn, reduce ongoing immunity and inflammation in the airways. We do not believe that a specific DC progenitor cell was depleted in the bone marrow by airways inflammation because over the 16 weeks, early hematopoietic progenitor and stem cells provide long lasting reconstitution of all hematopoietic cells [1-4]. Our previous studies of DCs differentiated *in vitro* but functionally assessed by adoptive transfer into naïve mice, and using the cyclooxygenase inhibitor, indomethacin [7], demonstrated that inflammation-associated prostanoids were involved in this process. Studies of DCs differentiated from the BM of mice implanted with subcutaneous slow-release PGE_2 pellets for 3 days suggested that PGE_2 was one of the prostanoids responsible [8]. Further, chimeric mice engrafted with BM cells from mice implanted with PGE_2-releasing pellets for 3 days have reduced DC-initiated immune responses [26]. Links between prostanoids, of which PGE_2 is the most abundant, and asthma are well recognized [27] and mechanistically, cyclo-oxygenase inhibition increases allergic inflammation in murine models of asthma [28, 29].

How inflammation-associated PGE_2 alters hematopoietic progenitor cells is a matter of ongoing research although it is clear that hematopoietic stem cells can respond via their EP4 receptor to PGE_2 [11, 19, 30-33]. PGE_2 can stimulate DNA methyltransferases [34] and here we show that the effect of inflammation in the airways on DC progenitors in the BM can be removed by treatment of OVA-sensitized mice with 5-Aza-dC, an inhibitor of DNA methylation. This result suggests PGE_2-dependent epigenetic (methylation) changes to cells unknown although it is of note that aging of hematopoietic stem cells is driven by a reversible epigenetic component rather than an increased DNA mutation burden [35]. We do not know the molecules whose transcription and expression in DCs may be affected by epigenetic change but candidates include NLRP10 [36]

or tetraspanin CD37 [37], both of which have been shown to contribute to the trafficking ability of activated DCs to draining nodes. Thus, we propose that inflammation in the airways, via PGE_2 production, has effects directly or indirectly on myeloid progenitor cells in the BM, such that *in vivo* differentiated DCs, upon activation, have reduced migratory properties. It is uncertain if myeloid progenitor cells in the BM are the only cell type altered by airways inflammation. Lymphocytes in the chimeric mice were not examined directly but responses dependent on lymphocyte activity versus DC function were assessed previously in chimeric mice engrafted with BM cells from mice implanted with PGE_2-releasing pellets for 3 days (PGE_2-chimeric mice). Administration of wild-type DCs to PGE_2-chimeric mice reversed the poor immune responses in those mice to levels detected in control-chimeric mice [26]. DCs are a cell type critical to initiation of an immune response; lymphocytes are important in the expression of the response but are not critical to defining the size of the immune response. PGE_2 also has effects on mature differentiated cells that may vary according to the microenvironment and other signals, but our study implicates an important role of PGE_2 on BM myeloid progenitor cells.

More than half a billion people worldwide suffer from inflammatory airway disorders, such as asthma. In addition to the individual suffering, these diseases place a great burden on healthcare systems and generate enormous costs for society. In this study in chimeric mice, we have identified a homeostatic response to airways inflammation that involves changes to hematopoietic progenitors in the BM. DCs differentiating from those BM cells are poorly immunogenic principally due to a reduced trafficking ability after activation. This is a pathway of inflammation control that, if supported in other experimental models, may be considered relevant to design of immunotherapies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the Telethon Kids Institute, University of Western Australia, at which the studies were conducted (Approval Number 235). This article does not contain any studies with human participants performed by any of the authors.

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Figure Legends

Fig. 1 Reconstitution of lymphoid organs of control- and EAAD-chimeric mice. Cells were examined in the BM, spleen and lymph nodes (pooled axillary, brachial, inguinal, mesenteric and cervical) of γ-irradiated C57BL/6J (CD45.2 alloantigen) mice reconstituted with BM cells derived from CD45.1 alloantigen mice ± EAAD. Reconstitution of lymphoid organs, (a) Number of cells/mouse, (b) %CD45.1, (c) %$CD19^+B220^+$ B cells, (d) %$CD3^+CD4^+$ T cells, and (e) %$CD11c^+$ cells, for BM, spleen and lymph nodes, respectively. The percentage of cells in the respective organs of age-matched, non-chimeric mice is shown by the horizontal line. Results for the control-chimeric mice (-EAAD) are shown by the solid line and circles (1 mouse/group/time point for each of 6 timepoints); for the EAAD-chimeric mice are shown by the dashed line and squares.

Fig. 2 Experimental allergic airways disease is reduced in EAAD-chimeric mice. Twenty-four h after the last OVA aerosol challenge for induction of EAAD, ADLN (mediastinal, tracheobrachial, and parathymic) of chimeric and age-matched non-chimeric mice were harvested, and BALF collected. (a) Number of ADLN cells/mouse, (b) Phenotype of ADLN cells (% of each cell type), (c) OVA-induced proliferation *in vitro* by ADLN cells measured by incorporation of ^{3}H-thymidine (CPM). Cells (10^5/well) were incubated for 96 h ± OVA (100 μg/mL) for 96 h. (d) Number of cells in BALF/mouse, and (e) Cell types in the BALF (%). For (f) and (g), serum was collected from chimeric and age-matched non-chimeric mice 6 h after the second OVA aerosol challenge. (f) OVA-specific serum IgE, (g) OVA-specific serum IgG1. For (a), (b), (d) and (e), the mean + SEM is shown for 7 mice (for (b), no error indicated for clarity); for (c), (f) and (g), mean + SEM for cells/sera from 5 mice/group. **P*<0.05 for comparison between chimeric mice (± EAAD), or non-chimeric mice.

Fig. 3 LPS-activated, PKH26-labelled DCs inefficiently traffic to the ADLN in EAAD-chimeric mice. PKH26 was given intranasally to mice, followed 8 h later with intranasal LPS. The mediastinal lymph node was collected from chimeric and age-matched non-chimeric mice 48 h after PKH26 exposure. (a) Number of cells in the mediastinal lymph node/mouse, without and with LPS exposure. Mean ± SEM for cells from 3 mice/group. **P*<0.05 for an LPS-induced increase in cell numbers. (b) Number of $CD11c^+$I-A/I-E^+ cells/mediastinal lymph node/mouse, (c) Number of $CD11c^+$I-A/I-E^+PKH26^+ cells/mediastinal lymph node/mouse. For (b) and (c), Mean + SEM for cells from 3 mice/group. **P*<0.05. (d) % $CD11c^+$ I-A/I-E^+ cells (boxed, % total cells shown) that were further examined in (e) for PKH26 and CD8α expression. (d) and (e) are

FACS plots of cells from a representative experiment for the mediastinal lymph node of chimeric mice (-EAAD and +EAAD) 48 h after intranasal PKH26 (40 h after LPS).

Fig. 4 FITC-labelled DCs inefficiently traffic to the SDLN in EAAD-chimeric mice. SDLN were harvested 16 h after topical FITC administration. (a) Cell yield/mouse in the SDLN. Mean ± SEM for cells from 3 mice/group. **P*<0.05 for a FITC-induced increase in cell numbers. (b) Plots of $CD11c^{+}$I-A/I-E^{hi} (upper gate) and $CD11c^{+}$I-A/I-E^{int} (lower gate) of cells from SDLN 16 h after exposure to FITC. (c) Histogram of cells expressing FITC from (b). Expression by $CD11c^{+}$I-A/I-E^{hi} cells is shown by the solid line, by $CD11c^{+}$I-A/I-E^{int} by the dashed line. (d) CD8α and FITC expression by the $CD11c^{+}$I-A/I-E^{hi} cells gated in (b). (e) CD45.1 and CD45.2 expression by $CD11c^{+}$I-A/I-$E^{hi}FITC^{+}$ cells gated in (b). For (b)-(e), results are shown for representative FITC-administered chimeric mice (± EAAD) and age-matched non-chimeric mice, as well as for non-chimeric mice that were not administered FITC (-FITC). In (f), the number of $CD11c^{+}$I-A/I-$E^{+}FITC^{+}$ cells/mouse in the SDLN. Mean + SEM for cells from 3 mice/group. **P*<0.05.

Fig. 5 Dendritic cells differentiated from the bone marrow of EAAD-chimeric mice have reduced priming ability. BM was taken from the control-chimeric mice (-EAAD) and the EAAD-chimeric mice 16 weeks after they received BM cells from the donor mice. The BM cells were cultured for expansion of DCs. As shown in (a), $CD11c^{+}$ cells were purified, loaded with DNBS and injected into the ears of naïve mice. Seven days later, the ears were challenged with DNFB. (b) Ear swelling after 24 h for naïve mice that were injected with cells from control-chimeric mice (-EAAD) and the EAAD-chimeric mice (+EAAD). Mean + SEM (n=8 ears/group), representative of 2 experiments. **P*<0.05.

Fig. 6 5-Aza dC prevents differentiation of dendritic cells with reduced priming ability from EAAD mice. OVA-in-saline aerosol was given to OVA-sensitized and -boosted mice 3 days before bone marrow harvest; 5-Aza dC (0.2 mg/kg), or diluted DMSO, was injected 1, 2 and 3 days before bone marrow harvest. The BM cells were cultured for expansion and purification of DCs, loaded with DNBS and injected into the ears of naïve mice. Seven days later, the ears were challenged with DNFB. Ear swelling after 24 h for naïve mice that were injected with cells from mice, ± EAAD, ± 5-Aza-dC. Mean + SEM (n=8 ears/group), representative of 2 experiments. **P*<0.05.

Table 1. Hematopoietic cells in blood from the chimeric mice 16 weeks after reconstitution.

Cell Type	*Non-chimeric*	*Control-chimeric (-EAAD)*	*EAAD-chimeric*
White blood cells ($x10^9$/L)	6.3±1.1[*]	13.3±1.0[†]	11.5±0.8[†]
Red blood cells ($x10^{12}$/L)	9.3±0.1	9.2±0.1	9.2±0.0
Platelets ($x10^9$/L)	1254±54.6	912.3±74.1	923.3±64.2
Neutrophils (%)	12.8±1.2	9.4±0.6	10.5±1.1
Lymphocytes (%)	79.6±1.0	84.1±1.3	82.1±1.9
Monocytes (%)	4.3±0.7	2.2±0.2	3.5±0.6
Eosinophils (%)	2.6±0.4	3.5±0.8	3.0±0.5
Basophils (%)	0.2±0.1	0.1±0.0	0.1±0.0

[*] Mean±SEM (n=4 mice)

[†] Greater than non-chimeric mice, $p<0.05$