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14. ABSTRACT

In vitro we tested our hypothesis that DCs from SRA KO mice would show altered migration and maturation, which would possibly explain the observation of enhanced allergy in the SRA KO mice. In detailed studies of random migration, chemotaxis to two different chemoattractants and surface marker maturation in response to two different stimuli (TNF and LPS) we found no difference in SRA deficient vs wild type dendritic cells. We made significant progress in establishing protocols for adoptive transfer studies that will allow testing of SRA deficient dendritic cells in vivo. It remains possible that the in vitro assays do not replicate the actual in vivo environment in which these differences are manifest. Hence, the focus of the remainder of the project will be to test these postulates using in vivo models. Both systemic and intratracheal adoptive transfer experiments will be used to fully test the role of the SRAs in modulating asthma susceptibility and severity in the mouse model. In addition, we made substantial progress in establishing inflammatory responses in SRA knockout mice in respone to oxidant air pollutants, which will allow direct testing of how air pollutants may modify SRA function in allergic responses in the coming period.

15. SUBJECT TERMS

asthma, allergy, scavenger receptors, dendritic cells

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INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Our central hypothesis is that lung macrophage scavenger receptors normally function to bind and clear inhaled allergens and pathogens, thereby preventing allergic responses and infections. The purpose of the project is to determine whether 1) decreased levels of SRAs (mediated by environmental stresses) increase susceptibility to asthma or pneumonia; and 2) therapy to increase or maintain normal levels of scavenger receptors will increase resistance to asthma and pneumonia. The scope of the research includes studies using in vivo mouse models (Aim 1), studies of the specific role of alveolar macrophages (Aim 2) and dendritic cells (Aim 3) and studies of the effects of pollutants on scavenger receptors (Aim 4).

Special Note: The review of last year's annual report included the critique that the report was insufficiently detailed. We agree, and appreciate this concern. In this year's report we have included a much greater level of detail in both methodology and results.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work.

For this period, our SOW identified three main tasks:

Task 2: **Determine role of SRAs on DCs in responses to inhaled allergen:** This work will test the hypothesis that DC SRAs act to down-regulate allergic immune responses. (Mos. 12-36):

- compare phenotype (cell surface markers) and function (migration, T cell stimulation, antigen uptake, signaling by SRA cross-linking) of DCs from wild-type (WT) and KO mice in vivo and in vitro.
- use DC deletion and adoptive re-constitution with wild-type or KO DCs to specifically test their ability to modulate responses.

Task 3: **Determine role of SRAs on AMs in responses to inhaled allergen:** This work will test the hypothesis that AM SRAs also mediate down-regulation of immune responses to allergens.
(Mos. 12-36):

- use adoptive transfer of wild-type or KO AMs to specifically test their roles in modulation of allergic responses in vivo
- use in vitro assays with wildtype or KO AMs to test their modulation of DC functions by nitric oxide (NO) or other candidate molecules

Task 4: **Determine role of SRA modulation by LPS/air pollutant on responses to inhaled allergen.** (Mos. 24-48)

- Expose allergic mice to lipopolysaccharide (LPS) to test the prediction that LPS-increased SRA leads to decreased asthma severity.

- Expose allergic mice to inhaled air pollutants to test the prediction that pollutant-generated oxidant stress down-regulates SRAs and thereby leads to increased asthma severity.
- Measure AHR and AI to gauge asthma severity, and use flow cytometry and RT-PCR to measure SRA expression on lung AMs and DCs. Use antioxidants to reverse effect of air pollutant exposures.

In vitro component of Task 2 & 3: While we have made good progress in some areas, we must also report some disappointing progress other areas, despite considerable effort.

As previously reported, to study dendritic cells we have learned and optimized in vitro culture protocols to grow bone-marrow derived dendritic cells from both wild-type and SRA knockout mice. This produces a large number of cells suitable for easy analysis of phenotype and function. We have confirmed purity of the cultured dendritic cells by immunolabeling with CD11c marker (>95% +).

Initial results with functional assays to compare DCs from wild-type and knockout mice were promising, as indicated in last year's report. However, as we repeated and expanded these analyses, we have consistently established an absence of significant differences in the wild-type vs. knockout groups. For example, to test whether scavenger receptors reduce cell motility (as observed in vivo as increased DC accumulation in lymph nodes in our asthma model), we have developed microscopic and live cell imaging assays to quantitate cell movement. The first assay uses modified Boyden migration chambers and results in counting of cells that move from an upper chamber to the lower chamber. The second assay measures random migration by tracing cell movement over 12-24 hours in a live cell system. The details of these assays and the results are presented next, followed by interim conclusions and discussion.

Dendritic cell isolation and differentiation:
Bone marrow progenitor cells were isolated from normal female wild-type or MARCO$^{-/-}$ adult (8-12 wk) Balb/c mice and cultured in 6-well tissue culture plates at 3e6/well in RPMI-10 (RPMI 1640 + 10% FBS + 1 mM l-glutamine + penicillin/streptomycin) with 20 ng/ml rGM-CSF (Peprotech) at 37° C, 5% CO_2. Media was changed every 2-3 d without disturbing the cells for 7 d. For motility and chemotaxis assays, on day 7-8 of culture, immature DC were replenished with media with or without maturation for 24 h using 1 µg/ml LPS, then harvested by pipetting and replated in assay dishes or wells. For surface receptor expression assays, immature cells were removed from plates by pipetting, replated in fresh media in low-adherence tissue culture dishes, and matured for 24-48 h with 20 ng/ml TNFα or 1 µg/ml LPS; cells were then removed by pipetting and stained for flow cytometry.

Random motility assay:
Immature and mature DC were harvested on day 8-9 of culture as indicated and stained with Hoescht (Molecular Probes) in normal saline + 10 mM HEPES + 0.5% BSA for 5 min at 37° C. Cells were then washed and adhered to glass-bottom tissue culture plates (MatTek) coated with 50 µg/ml fibronectin (Sigma) for 4 h at 37° C, 5% CO_2 in RPMI-10 + 10 mM HEPES + 20 ng/ml rGM-CSF. Cells were then imaged at 5 min intervals for 16 h using a Nikon confocal microscope fitted with an environmental chamber at 37° C.

Cell tracks were generated by tracking nuclei positions using the MTrackJ plugin (Erik Meijering, University Medical Center, Rotterdam, Netherlands) for ImageJ (NIH). Graphs and analysis were performed using the Chemotaxis Tool plugin (Ibid) for ImageJ.

Chemotaxis:

Immature and mature DC were harvested on day 8-9 of culture as indicated and resuspended in RPMI-10 at 1e7/ml, then 100 µl cell suspension was placed into the top well of 8 µm pore transwells (BD Falcon) in 24-well tissue culture plates (BD Falcon) containing 0.5 ml RPMI-10 ± 0.1-100 nM CCL21, 50 ng/ml MIP-1α, or 100 ng/ml MCP-1 (all chemokines obtained from Peprotech) in triplicate wells per condition. Cells were allowed to migrate for 4 h at 37° C, 5% CO_2; transwells were then removed and cells that had migrated to the lower well were harvested using 2 mM EDTA. Migrated cells were counted using flow cytometric cell counting, and percent migrated cells was determined for each well using cell counts obtained from parallel wells containing cells without the transwell inserts. Chemotactic indices were calculated by normalizing all data relative to the percentage of migrated cells (matched type and maturation state) in wells with no chemokine.

Results:

No significant differences were observed in DC migration, either in random movement in the absence of stimulation or in chemotaxis in response to CCL21 or MIP-1α. This suggests that MARCO does not act as a molecular "brake" on DC movement from the lung to the lymph nodes.

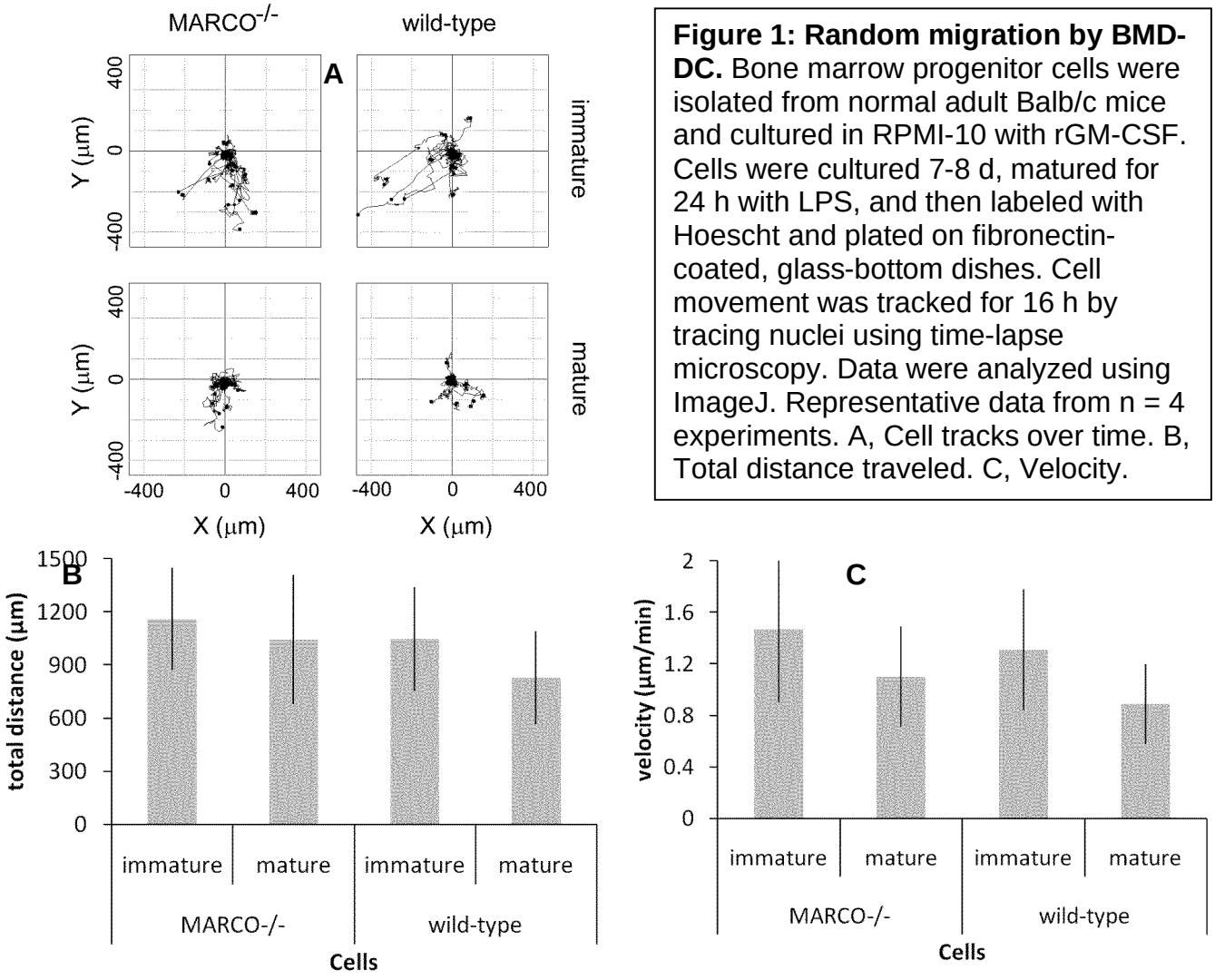


Figure 1: Random migration by BMD-DC. Bone marrow progenitor cells were isolated from normal adult Balb/c mice and cultured in RPMI-10 with rGM-CSF. Cells were cultured 7-8 d, matured for 24 h with LPS, and then labeled with Hoescht and plated on fibronectin-coated, glass-bottom dishes. Cell movement was tracked for 16 h by tracing nuclei using time-lapse microscopy. Data were analyzed using ImageJ. Representative data from n = 4 experiments. A, Cell tracks over time. B, Total distance traveled. C, Velocity.

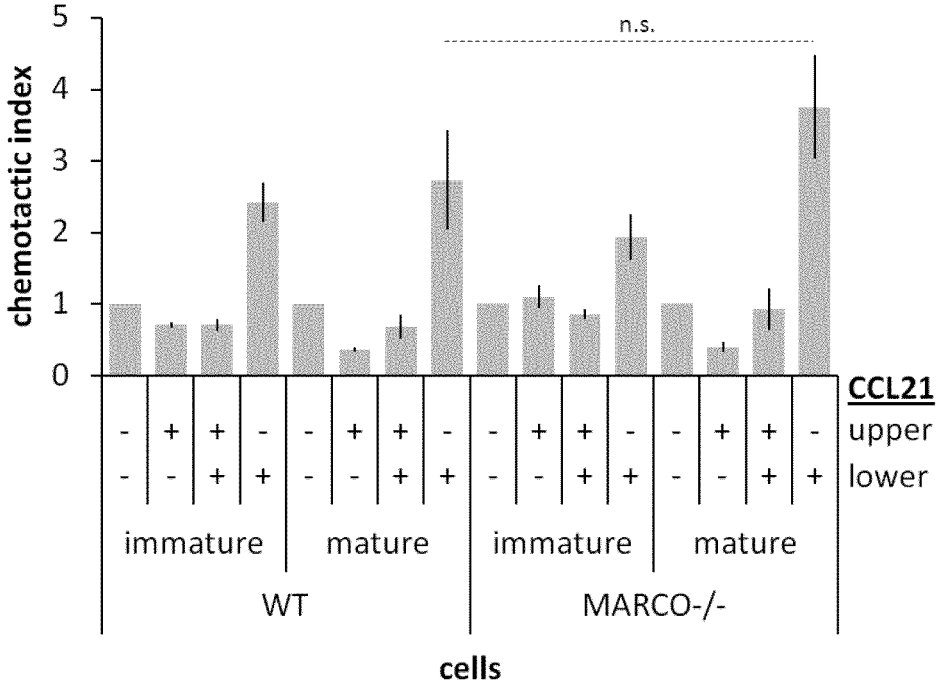


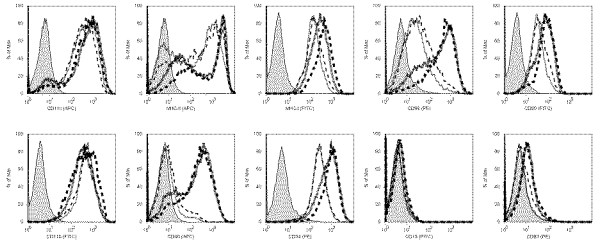
Figure 2: Chemotaxis by BMD-DC. Bone marrow progenitor cells were isolated from normal adult Balb/c mice and cultured in RPMI-10 with rGM-CSF. Cells were cultured 7-8 d, matured for 24 h with LPS, then placed in the upper well of 8 μm transwells with (+) or without (-) 100 nM CCL21 in the upper and lower wells as indicated, and allowed to migrate for 4 h at 37° C. Transwells were then removed and cells that had migrated to the lower well were harvested and counted using flow cytometric cell counting. The percent migrated cells were determined using cell counts obtained from parallel wells containing cells without the transwell inserts, and chemotactic indices were calculated by normalizing all data relative to the percentage of migrated cells in wells with no chemokine. Cumulative data are mean ± standard error of n = 8 experiments. All values obtained from wells with CCL21 in the lower well only were significant compared to wells with no chemokine. N.S., not significant ($p > 0.3$).

We hypothesized that MARCO alters DC migration by affecting DC motility, DC maturation, or both. In parallel to the evaluation of migration, results of which reported above, we examined the effects of MARCO on DC maturation in response to two maturation stimuli, TNFα and LPS, using flow cytometry to measure changes in surface marker expression prior to and following maturation caused by these agents.

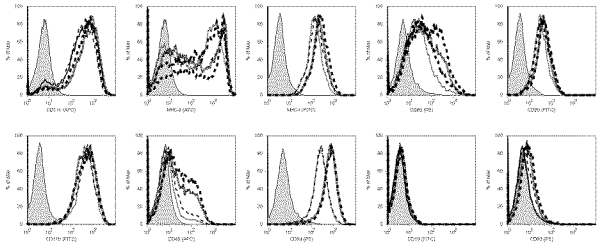
Although surface expression of key DC maturation markers (CD80, CD86, MHC-II), adhesion molecules (CD11b, CD11c, CD54), and antigen presentation/costimulation proteins (CD40, CD80, CD86, MHC-I, MHC-II) was increased in LPS- and TNFα-matured DC, no significant differences were observed in their expression on MARCO$^{-/-}$ versus wild-type DC. We next report the methods used and the results of these studies.

Cell surface receptor expression by flow cytometry:
Immature or mature DC were harvested on day 9 of culture, resuspended in Facs Buffer (PBS + 10 mM HEPES + 2 mM EDTA + 0.5% BSA), and stained for surface markers using specific labeled antibodies against CD11c, CD11b, CD19, CD40, CD54, CD80, CD83, CD86, MARCO, MHC-I, and MHC-II, or isotype controls (all antibodies obtained from eBioscience). Cells were then washed, fixed, and cellular fluorescence measured using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

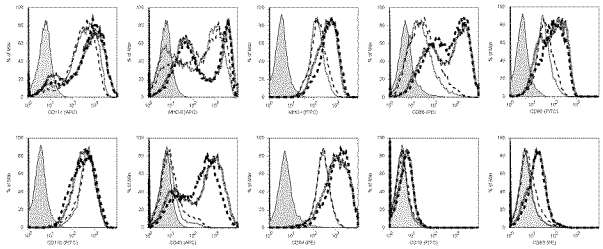
24h LPS



24h TNFα



48h LPS



48h TNFα

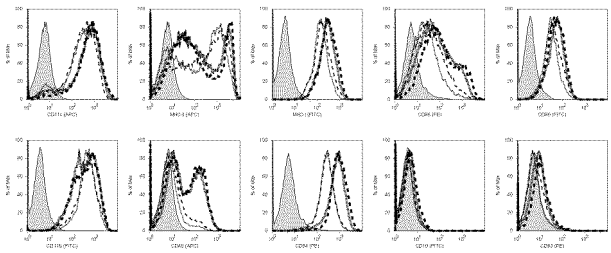


Figure 3: Surface receptor expression by BMD-DC. Bone marrow progenitor cells were isolated from normal adult Balb/c mice and cultured in RPMI-10 with rGM-CSF. Cells were cultured 7 d, matured for 24-48 h with TNFα or LPS, and then stained for surface markers using specific labeled antibodies against CD11c, MHC-II, MHC-I, CD86, CD80, CD11b, CD40, CD54, CD19, CD83, or isotype controls. Representative data from n = 4 experiments. Shaded line, isotype staining of BMD-DC. Thin dotted or dashed lines, immature cells. Thick dotted or dashed lines, LPS- or TNFα-matured cells. Dotted lines, MARCO$^{-/-}$ DC. Dashed lines, wild-type DC.

In Vitro Summary and Discussion:

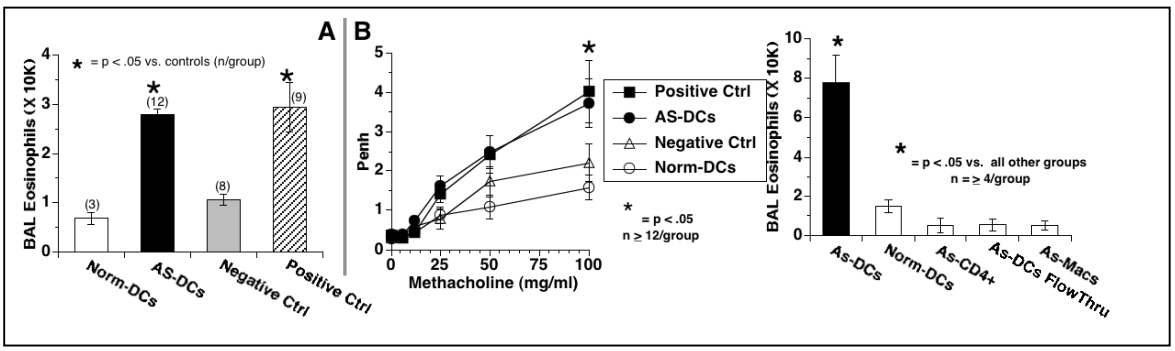
Further studies using labeled MARCO$^{-/-}$ versus wild-type DC instilled into wild-type versus MARCO$^{-/-}$ mouse airways may reveal a difference in migration rate *in vivo* of cells loaded with antigen and/or matured *in vitro*, and these studies will be conducted

In vivo component of task 2-4. We have made good progress in a number of experimental areas related to the in vivo components of tasks 2-4. First, we have established effective adoptive transfer protocols using either primary DCs harvested from spleen or bone-marrow derived DCs. These protocols show clear acquisition of asthma susceptibility in otherwise normal recipients of DCs harvested from 'asthmatic' donor mice, but not from normal non-asthmatic DCs. The development of these protocols is a key step, especially the ability to cause increased asthma susceptibility using bone-marrow derived DCs. The latter allows us to more easily prepare the large number of DCs from wild-type and knockout mice needed for the adoptive transfer into sufficient replicate animals. A second area of good progress has been in the analysis of environmental exposures on airway responses and the role of MARCO and SRA in this response (Task 4). We have begun the LPS exposures with promising results, and have also observed relevant differences in responses to oxidant air pollutant exposure in MARCO/SRA knockout mice exposed to ozone. These methods and results are presented next.

Adoptive Transfer of Primary DCs

We used magnetic beads (Miltenyi) to isolate splenic DCs from female mice sensitized with OVA and aerosol challenged (asthmatic) or normal controls. The purified CD11c+ DCs were injected i.p. (2 X 10^5) into normal non-allergic mice. The next day, recipient mice were then subjected to the 'intentionally suboptimal' protocol using OVA (single ip injection rather than two, followed by 3 days of aerosol challenge 10 days after injection). As shown in Fig 4, the main finding is that an asthma phenotype developed after the adoptive transfer of DCs purified from 'asthma-susceptible' mice, but not after adoptive transfer of DCs from normal pups. Important negative controls include lack of effect by injection of splenic CD4+ T cells, macrophages or other cells that flow through the isolation column (Figure 4C).

Figure 4: Adoptive transfer of DCs. Normal mice received splenic CD11c+ DCs from asthmatic (AS-DCs) or normal (Norm-DCs) donors, followed by the 'intentionally suboptimal' protocol. Recipients of AS-DCs, but not Norm-DCs, showed allergic inflammation (A) and Penh responses (B) comparable to the positive control (standard OVA sensitization). Adoptive transfer of other splenocytes or flow-through non-DC cells had minimal effect (C).



Adoptive Transfer of Cultured DCs.

To facilitate adoptive transfer of DCs from SRA knockout mice, we sought to develop a source from bone-marrow derived DCs. Moreover, since cytokine skewing of DC towards Th1- versus Th2-inducing DC (DC1 and DC2, respectively) has been studied by many laboratories, we also investigated this protocol to increase our chances of testing the influence of SRA receptors in a DC2 type population.

Dendritic cell isolation and differentiation:
We followed a protocol designed to produce DC0, DC1, and DC2 subtypes of DCs. Bone marrow progenitor cells were isolated from normal adult (8-12 wk) Balb/c mice and cultured in 6-well tissue culture plates at 3e6/well in RPMI-10 (RPMI 1640 + 10% FBS + 1 mM l-glutamine + penicillin/streptomycin) with 20 ng/ml rGM-CSF + 20 ng/ml rIL-3 (Peprotech) at 37° C, 5% CO_2. To these cultures were added no cytokines (DC0), 20 ng/ml IFNγ + 20 U/ml rIL-12 (DC1), or 20 ng/ml rIL-4 (DC2). Media was changed every 2 d without disturbing the cells. On day 4 of culture, loosely adherent cells were removed by pipetting, washed twice in PBS, and counted. DC were resuspended in PBS at 2e6 live cells/ml (by trypan blue exclusion), where dead cells typically constituted less than 20% of total cells. DC were adoptively transferred to normal P4 Balb/c pups by i.p. injection of 100 µl cells (2e5 DC). Remaining DC were resuspended in Facs Buffer (PBS + 10 mM HEPES + 2 mM EDTA + 0.5% BSA) and stained for surface markers using specific labeled antibodies against CD11c, CD11b, CD40, CD80, CD86, MHC-I, and MHC-II, or isotype controls (all antibodies obtained from eBioscience).

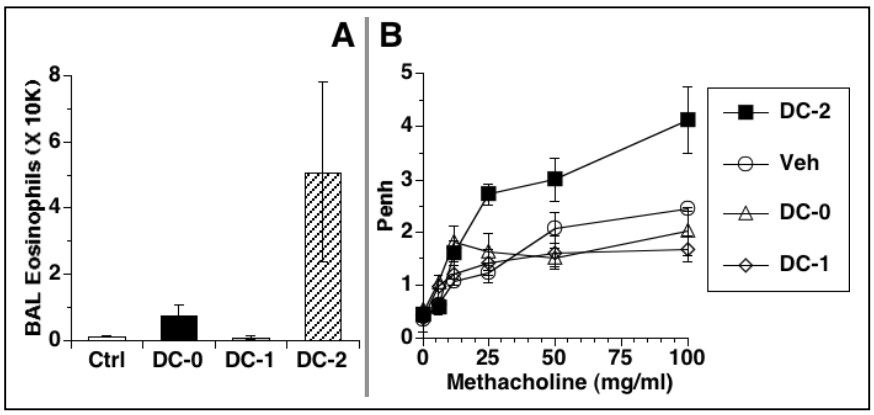


Figure 5: Adoptive transfer of in vitro bone marrow-derived dendritic cells. Normal mice received DCs differentiated to favor DC0, DC1 or DC2 phenotypes, and were subjected to the 'intentionally suboptimal' protocol. Recipients of DC2, but not DC0 or DC1 cells, showed enhanced AI (A) and Penh responses (B), consistent with re-creation of the 'asthma-susceptible' DC phenotype.

Task 4: Modulation of SRAs and Air Pollution Responses

This task includes plans to expose allergic mice to lipopolysaccharide (LPS) to test the prediction that LPS-increased SRA leads to decreased asthma severity. In addition, another goal is to expose allergic mice to inhaled air pollutants to test the prediction that pollutant-generated oxidant stress down-regulates SRAs and thereby leads to increased asthma severity.

As planned in the overall schedule, we have begun work on this task. For air pollution studies, we have made progress using ozone as a model oxidant . The data using ozone support the predicted effects of solid particulates, and studies of those agents are underway.

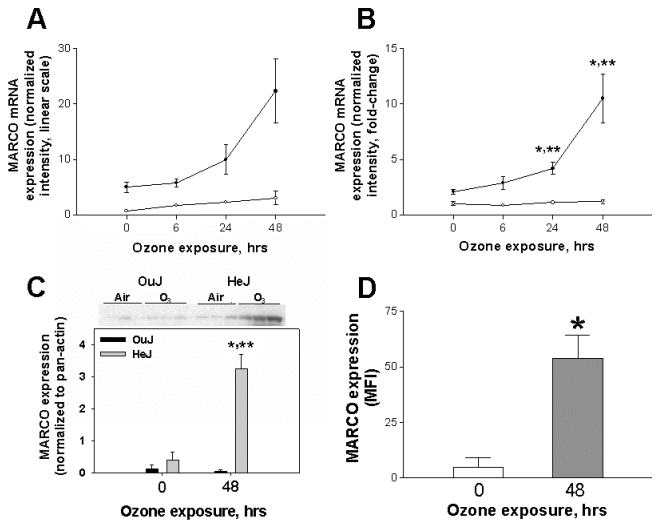


Figure 6 – Ozone up-regulates MARCO in lungs from ozone-resistant HeJ mice. HeJ or congenic ozone-sensitive OuJ mice were exposed to 0.3ppm ozone for up to 48hrs. Microarray analysis (**A**) and RT-PCR (**B**) were performed on total RNA isolated from lung samples and shows increased MARCO mRNA expression in HeJ mice (●) compared with OuJ mice (○); *P<0.05 compared with OuJ ozone at same timepoint, **P<0.05 compared with air control. Western blot analysis of lung tissue obtained after 48 hours of ozone exposure also shows increased MARCO protein expression (**C**); *P<0.05 compared with OuJ ozone, **P<0.05 compared with air control. Ozone up-regulates MARCO on the surface of alveolar macrophages of C57BL/6 mice exposed to 0.3ppm ozone for 48hrs, as shown by increased fluorescence after flow cytometric analysis (**D**); *P<0.05 compared with air control; results shown are representative of three independent experiments. MFI = mean fluorescence intensity.

As shown in Fig. 6, oxidant pollutant exposure increased MARCO expression at the mRNA level, using both array and PCR analysis, and at the protein level (Western blot and cell surface analysis). Additional experimentation has shown that both MARCO and SRA KO mice demonstrate increased acute inflammation in response to the mild ozone challenge used here, most likely linked to defective clearance of pro-inflammatory oxidized phospholipids in the absence of the scavenger receptors.

We have begun studies using exposure to solid air pollutants. As shown in Fig. 7 below, we find similar enhanced acute inflammation in MARCO deficient mice exposed to air pollution samples. However, this difference is only evident when the soluble component of the air pollution particles is instilled, a finding that is under current further investigation.

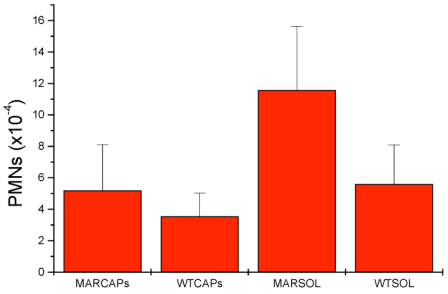


Fig. 7. Comparison of acute inflammation after intratracheal instillation of concentrated air particles (CAPs, 50 ug/mouse) or the soluble component equivalent (supernatant of CAPs suspension). The results show increased inflammation in MARCO -/- mice treated with the soluble fraction (MARSOL group), and similar acute PMN levels in other groups. (N= 4 mice/group)

KEY RESEARCH ACCOMPLISHMENTS:

- Completed experiments to compare random migration and directed chemotaxis in dendritic cells from wild-type and MARCO-deficient mice
- Completed experiments to compare cell surface immunophenotype before and after in vitro maturation with TNF and LPS in dendritic cells from wild-type and MARCO-deficient mice
- Established first phase of adoptive transfer experiments in vivo to test ability of wild-type vs. knockout dendritic cells to alter susceptibility to asthma
- Established in vitro system to generate TH2 vs TH1 skewing dendritic cells to more efficiently test ability of wild-type vs. knockout dendritic cells to alter susceptibility to asthma in adoptive transfer experiments
- Confirmed predicted increased sensitivity of SRA deficient mice (MARCO and SRA) to inflammation caused by oxidant pollutants with gaseaous ozone and begun similar studies with solid phase air pollution particles.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

We expect to include the ‘negative’ data regarding comparison of dendritic cell function in a manuscript that reports the totality of findings, based on expected progress this coming period.

CONCLUSION:

We are satisfied with progress in certain aspects of the project. These include the development of the protocol for adoptive transfer studies, and the directed experimentation using these systems to analyze SRA contribution to asthma susceptibility is underway. Similarly, we have established conditions to increase SRA expression with oxidant exposure and to test effects on asthma severity and susceptibility and these protocols are also underway. These include the use of ‘pre-skewed’ DCs developed in vitro as described which will provide a direct test of SRA function in this area.

We are less satisfied with the difficulties encountered in comparison of DC function. Despite considerable efforts, and multiple experiments only summarized here, we did not find evidence to support our original hypothesis that DCs from SRA KO mice would show altered migration and maturation, which would possibly explain the observation of enhanced allergy in the SRA KO mice. It remains possible that these in vitro assays do not replicate the actual in vivo environment in which these differences are manifest. Hence, the focus of the remainder of the project will be to test these postulates using in vivo models. Both systemic and intratracheal adoptive transfer experiments will be used to fully test the role of the SRAs in modulating asthma susceptibility and severity in the mouse model.

REFERENCES: None

APPENDICES: None