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Carbonic Anhydrase IV Is Expressed on IL-5–Activated Murine Eosinophils

Ting Wen,* Melissa K. Mingler,* Benjamin Wahl,‡ M. Eyad Khorki,* Oliver Pabst,* Nives Zimmermann,* and Marc E. Rothenberg*

Eosinophilia and its cellular activation are hallmark features of asthma, as well as other allergic/Th2 disorders, yet there are few, if any, reliable surface markers of eosinophil activation. We have used a FACS-based genome-wide screening system to identify transcriptional alterations in murine lung eosinophils recruited and activated by pulmonary allergen exposure. Using a relatively stringent screen with false-positive correction, we identified 82 candidate genes that could serve as eosinophil activation markers and/or pathogenic effector markers in asthma. Carbonic anhydrase IV (Car4) was a top dysregulated gene with 36-fold induction in allergen-elicted pulmonary eosinophils, which was validated by quantitative PCR, immunohistochemistry, and flow cytometry. Eosinophil CAR4 expression was kinetically regulated by IL-5, but not IL-13. IL-5 was both necessary and sufficient for induction of eosinophil CAR4. Although CAR4-deficient mice did not have a defect in eosinophil recruitment to the lung, nor a change in eosinophil pH-buffering capacity, allergen-challenged chimeric mice that contained Car4−/− hematopoietic cells aberrantly expressed a series of genes enriched in biological processes involved in epithelial differentiation, keratinization, and anion exchange. In conclusion, we have determined that eosinophils express CAR4 following IL-5 or allergen exposure, and that CAR4 is involved in regulating the lung transcriptome associated with allergic airway inflammation; therefore, CAR4 has potential value for diagnosing and monitoring eosinophilic responses. The Journal of Immunology, 2014, 192: 5481–5489.
heterozygous matings were used in all experiments. In some initial validation experiments, BALB/c mice were subjected to CAR4 FACS staining, with no eosinophil CAR4 expression difference found between the two strains. All mice used in this study were housed in specific pathogen-free conditions at Cincinnati Children’s Hospital Medical Center under Institutional Animal Care and Use Committee-approved protocols. All mice were housed in a room with an ambient temperature of 22°C and a 12-h light cycle.

**Genome-wide microarray analysis on sorted eosinophils and quantitative PCR validation**

Tissue eosinophils were isolated from the perfused lungs, as previously described (14). Briefly, eosinophils were sorted as DAPI + CD11b+Siglec-F+CD45 CD14-CD8α CD19+ B220+ side scatterhigh cells from C57BL/6 animals using the FACSaria (BD Biosciences). Total RNA from sorted eosinophils (purity, 95–99%) was extracted by a standard TRIzol (Life Technologies) RNA isolation and subsequently with an RNeasy Mini Kit (QIAGEN). RNA integrity was validated by the Agilent 2100 Bioanalyzer (Agilent Technologies). Eosinophil mRNA was amplified and labeled with the WT-Ovation Pico RNA Amplification System (NuGen) and subjected to the GeneChip Mouse Gene ST 1.0 Array chip (Affymetrix). Following probe preparation and hybridization, data were analyzed using the Genespring GX 11 software (Agilent Technologies). Differential expression between activated lung eosinophils and saline controls was identified by upper 80th percentile expression, >2.0-fold change, and p < 0.01 with a false discovery rate (FDR) correction. For the lung tissue microarray following bone marrow transplantation, Affymetrix Mouse Gene ST 2.0 Array chips and a filter of >1.4-fold change and a Westfall–Young–corrected p < 0.001 were used. For real-time PCR confirmation, the same mRNA sample used for microarray analysis was reverse transcribed with the iScript cDNA Synthesis Kit (170-8891; Bio-Rad, Hercules, CA), and real-time PCR was performed with the IQ5 Real-Time PCR Detection System (Bio-Rad) using a pair of primers spanning exons 1 and 2 of the Car4 transcript (forward: 5′-CATG-CAGGCTCTCTTCGCTCT-3′; reverse: 5′-TTCTGAATCTCATAGCACCA-GCC-3′), resulting in an 83-bp amplicon.

**Flow cytometry analysis of eosinophil surface expression and signaling**

Polychromatic flow cytometry compensation was preset in pilot studies for up to five channels on a FACSCanto II flow cytometer (BD Biosciences). Abs/dyes used in this study were purchased from BioLegend (CD11c-Pacific Blue N418, human Siglec F 7C9, human CD11b ICRF44, human CD45 2D1), Invitrogen (LIVE/DEAD Violet dye), BD Biosciences (CD45- FITC 30-F11, Siglec F-PE E50-2440, CD11b-PECY7 M7/10, Gr-1 PerCP/Cy5.5 RB6-8C5), and R&D Systems (msCAR4, AF2414). Staining was performed on ice for 30 min in staining buffer (0.5% BSA, 0.01% NaN3 in 1X HBSS) with manufacturer-suggested titers. A secondary staining with the anti-goat AF488 was used for all CAR4 staining. Stained cells were resuspended and analyzed to assess the BD FACSCanto II flow cytometer. In some experiments, stained cells were fixed before being subjected to FACS analysis in a controlled fashion. Raw flow cytometry data were analyzed by FlowJo software (TreeStar). For phospho-FACS, eotaxin-1 (25 ng/ml) stimulation (2 min) was used for p44/42 MAPK activation assessment. Cells were immediately fixed by 2% formaldehyde at 4°C for 10 min, followed by ice-cold methanol permeabilization for 30 min. Specific Thr202/Tyr204 p44/42 Ab (E10), #4375; Cell Signaling Technology) was used together with previously mentioned eosinophil markers to assess eosinophil MAPK activation. For flow-imaging analysis of bronchoalveolar fluid (BALF) eosinophils, stained cells were fixed with 2% formaldehyde and run through the ImageStreamX system (Amnis), following the manufacturer’s instructions, as previously reported (14).

**CAR4 immunohistochemistry**

A consistent lobe (lower right) of mouse lung tissue was fixed in 10% formalin under negative pressure for 6 h before regular paraffin embedding and sectioning procedures. After citric buffer Ag retrieval, the 5-μm sections were incubated with either anti-CAR4 polyclonal Ab (AF2414; R&D) or IgG control (AB-108-C; R&D) in 1% rabbit serum overnight at 4°C, followed by incubation with biotinylated rabbit anti-goat secondary Ab for 1 h at room temperature. The slides were developed by a VECTASTAIN Elite ABC Kit (Vector Laboratories), following the standard manufacturer’s protocol, and counterstained by hematoxylin.

**Bone marrow–derived eosinophils**

Whole bone marrow was cultured using a modification of previously described methods (15). During the 14-d culture, the culture media were supplemented with FLT3 and stem cell factor at 100 ng/ml (PeproTech) for the first 4 d and IL-5 at 10 ng/ml (PeproTech) for the following 10 d. Results from H&E staining and FACS analysis indicated that the purity of eosinophils was >90% at day 14.

**Experimental asthma induction**

For the OVA model, mice were sensitized with 100 μg OVA/Alum twice, 14 d apart. For OVA intranasal challenge, OVA (100 μg in 50 μL saline) was administered for 3 consecutive days. Mice were sacrificed 48 h after the third OVA challenge for eosinophilia assessment. For the Aspergillus model, 100 μg Aspergillus fumigatus (GREER, Lenoir, NC) was dissolved in sterile saline and administered intranasally every other day into wild-type or IL-5–transgenic mice (BALB/c or C57BL6); a total of five challenges were given unless otherwise noted in the results. For the CC10–IL-13 transgenic model, IL-13 expression is driven by the lung epithelium promoter CC10 under the control of doxycycline (16), and the asthma model was established after the mice were treated with doxycycline for 4 wk.

**Bone marrow transfer experiments**

CD45.1 recipient mice received sequential doses of irradiation, 7 Gy and then 4.75 Gy, before a bolus transfer of 1.1 × 10⁸ RBC-lysed donor CD45.2 Car4+ or Car4− whole bone marrow cells. CD45.1 recipient mice were fed with food containing doxycycline for 2 wk thereafter and periodically checked for CD45.2 blood CD11b+ cell engraftment efficiency by FACS. Allergen challenge started when the CD11b+ engraftment was >99% for animals with 3 wk after the transfer.

**Lactate-induced acidification**

Car4+ and Car4− bone marrow–derived eosinophils were loaded with 5 μM pH-sensitive dye SNARF-1AM and then incubated with 15 mM lactate for 20 min. Intracellular pH (pHi) was determined by flow cytometry, comparing with eosinophils whose pH was clamped with nigericin, as previously described (17).

**Statistical analysis**

Statistical significance was analyzed using a two-tailed Student t test in all instances, unless otherwise noted. Data are graphed as mean ± SEM. A p < 0.05 was deemed significant in experiments not involving a microarray, whose criteria are specifically mentioned in Materials and Methods and in Results.

**Results**

**Eosinophil RNA microarray identifies a unique transcriptome with Car4 as a lead transcript**

After lung perfusion and collagenase digestion, lung tissue eosinophils were FACS sorted for DAPI−CCR3+Siglec-F+CD45+ CD8α−CD19− B220− and SSChigh events, from saline-challenged lung (Fig. 1A, upper panel) and OVA-challenged lung (Fig. 1A, lower panel). Eosinophil sorting purity (>95–99%) in saline and OVA samples was confirmed by back-running the sorted samples with an identical gating strategy (Fig. 1A). Microarray analysis of these isolated cells led to the identification of a total of 82 genes that were differentially expressed, 78 upregulated and 4 downregulated, in eosinophils from OVA-challenged versus saline-challenged lungs (Fig. 1B, Supplemental Table I). Among the most induced genes, Car4 mRNA was robustly upregulated by 36-fold (p < 0.0001, FDR-corrected p = 0.01) in eosinophils following OVA challenge (Fig. 1C). We next confirmed the Car4 mRNA upregulation by quantitative PCR from the same sorted RNA sample, verifying a dramatic 165-fold increase after normalizing to Gapdh (p < 0.0001, Fig. 1D). To assess the potential involvement of other CARs, we also collectively analyzed the differential expression level of all 15 CAR family member transcripts embedded on the array and found that eosinophil Car4 upregulation was unique among all of the CAR members screened (Fig. 1E).

**Lung eosinophil CAR4 surface expression is driven by Th2 inflammation**

We aimed to validate CAR4 expression at the protein level on both BALF and lung tissue eosinophils following allergen (A. fumigatus) challenge (18), in which eosinophils are a major population in...
BALF and lung tissue after five allergen challenges. Car4\textsuperscript{+/+} and Car4\textsuperscript{-/-} mice were used to test the specificity of Ab staining, whereas polychromatic FACS readily identified a pronounced eosinophil population (typically \(\leq80\%\) of BALF cells) by SSC\textsuperscript{high} Siglec-F\textsuperscript{-}CD11b\textsuperscript{-}Gr-1\textsuperscript{-}CD11c\textsuperscript{-} serial gating (Fig. 2A), resulting in \(>99\%\) purity from BALF (Figs. 1A, 2F). We therefore used...
a similar gating strategy to study eosinophil populations by FACS throughout this study. Gated on BALF eosinophils, CAR4 staining controlled by nonspecific IgG staining revealed robust CAR4 surface staining only on Car4<sup>+</sup> BALF eosinophils, but not on Car4<sup>-</sup> BALF eosinophils and not on blood eosinophils from the same animal of either genotype (Fig. 2B). We identified a comparable CAR4 expression pattern in the OVA-challenging model that was originally used in the sorting-based microarray (Supplemental Fig. 1A). We also assessed CAR4 surface expression on major leukocytes in the lung, BALF, and blood. Of note, unlike lung eosinophils, which expressed surface CAR4 only following allergen challenges, lung macrophages (under homeostasis or allergen challenge), but not lymphocytes and neutrophils, also expressed CAR4 on their surface independent of allergic inflammation (Supplemental Fig. 2A).

We next examined CAR4 surface expression on lung tissue eosinophils from wild-type and Il5 transgenic mice and identified a similar heterogeneous expression pattern, with the latter exhibiting more robust CAR4 expression (Fig. 2C), suggesting a role for IL-5 in CAR4 induction. As IL-13 is sufficient to elicit extensive lung eosinophilia (19), we used the CC10–IL-13 transgenic system (16) to assess the role of IL-13 in eosinophil CAR4 induction. Of note, IL-13 overexpression failed to induce eosinophil CAR4 expression (Fig. 2D) despite the presence of extensive eosinophilic lung inflammation.

Notably, heterogeneous expression of CAR4 was consistently observed after five allergen challenges, typically resulting in a double-peak histogram, with ~50% of the eosinophils remaining CAR4 negative (CAR4<sup>-</sup>). FACS imaging micrographs identified surface labeling of CAR4 and the coexistence of CAR4<sup>+</sup> and CAR4<sup>-</sup> eosinophils in the BALF from mice with allergic inflammation (Fig. 2E). FACS sorting of CAR4<sup>+</sup> and CAR4<sup>-</sup> eosinophils demonstrated that the CAR4<sup>+</sup> eosinophils contained 20-fold higher Car4 transcript than did CAR4<sup>-</sup> eosinophils, de-
spite comparable morphology (Fig. 2F). In addition, heterogeneous CAR4 expression on eosinophils did not correlate with eosinophil physical parameters (forward and side scatter) or a panel of known eosinophil/granulocyte surface markers related to key functions (CCR3, Siglec-F, CD11b, CD11c, Gr-1, αE, αβ1, β2 and β7 integrin, and l-selectin/CD62L, IL-5R/CD125, CD69, CD86, CD25, and intracellular IL-4) (Supplemental Fig. 1B). In addition, an unaltered eotaxin-CCR3 signaling strength was found on the basis of p44/42 MAPK activation capacity of CAR4+ and CAR4− eosinophils (Supplemental Fig. 1C, Car4+/− sample used).

We also examined CAR4 distribution in the normal and allergic lung by immunohistochemistry (IHC). In the OVA asthma model, whereas CAR4 was positive in the alveolar area regardless of treatment, CAR4-positive infiltrating inflammatory cells were found in OVA lung only in the anatomical regions traversing blood vessel and airway (the asthmatic pathogenic zone), an area clearly CAR4 negative in the saline control (Fig. 3A, ×200). At higher power magnification (×400), in a different asthma model (induced by A. fumigatus Ag), CAR4-positive events were found only in the CAR4+/− lung stained with CAR4, not in the CAR4−/− lung, and not in the IgG control staining in both cases (Fig. 3B). A high-power micrograph focusing on the alveolar region revealed CAR4 expression on lung macrophages and eosinophils (Fig. 3C).

**IL-5 is necessary and sufficient for eosinophil CAR4 surface induction**

The enhanced CAR4 expression in eosinophils from IL-5 transgenic mice led us to hypothesize that IL-5 is a key driver of CAR4 induction during allergic airway inflammation. To test this hypothesis, the ability of IL-5 to modulate CAR4 expression during eosinophil development was assessed (15). Indeed, CAR4 on de-
veloping eosinophils was kinetically induced by IL-5 in vitro, reaching ~80% CAR4 positive about day 20 (Fig. 4A, gated eosinophils shown). In addition, delivery of exogenous IL-5 (1 μg) into the peritoneal cavity of wild-type mice induced CAR4 upregulation on eosinophils (Fig. 4B, gated eosinophils shown). Finally, we also established allergic inflammation in allergen-challenged Il5−/− and Il5+/− mice and found that BALF eosinophils from Il5−/− mice were largely CAR4− (Fig. 4C), whereas eosinophils from Il5+/− mice were mostly CAR4+. Of note, CAR4 demonstrated more homogeneous staining under these experimental conditions, likely the result of administering seven allergen challenges. To further determine whether IL-5 was necessary and sufficient, we exposed BALF cells from Il5−/− allergen-challenged mice to exogenous IL-5 for 72 h (20 ng/ml) and compared the eosinophil CAR4 expression before and after IL-5 exposure. IL-5 treatment indeed induced CAR4 expression (Fig. 4D). Compared with Car4+/+ eosinophils, Car4−/− bone marrow-derived eosinophils did not exhibit a difference in differentiation in terms of specific surface marker expression, yield, or morphology (Supplemental Fig. 1D and data not shown).

Biochemical assessment of putative Car4 functions on eosinophils

Considering the reported functions of CAR4 (11, 12, 20), we examined the cellular buffering capacity by bone marrow–derived eosinophils. Cellular pH buffering capacity was readily observed for eosinophils, with Car4+/+ and Car4−/− eosinophils having similar activity (Fig. 5A). A well-established function of membrane-bound carbonic anhydrases is to enable lactate transport into cells by buffering extracellular pH following lactate–H+ cotransport (21, 22). Because readout of this process is intracellular acidification following lactate treatment, the pH in cultured eosinophils was monitored by a pH-sensitive dye following addition of lactate. Although the intracellular acidification was readily observed, no difference in intracellular acidification was found between CAR4+/+ and CAR4−/− eosinophils (Fig. 5B).

A novel epithelium-modulating function of eosinophil CAR4

We next investigated whether CAR4 expression on eosinophils could affect eosinophil migration and levels in the allergic airway. For this purpose, we allergen challenged irradiated CD45.1 bone marrow recipient mice that had been adoptively transferred with either Car4+/+ or Car4−/− bone marrow from CD45.2 donor mice. CD45.2 donor engraftment (CD11b+) was >99%, as assessed by the presence of CD45.2+CD11b+ granulocytes, including eosinophils, in the blood of CD45.1 recipients (Supplemental Fig. 2B); thus, the results can be attributed to hematopoietic cells rather than lung tissue cells (such as endothelial cells, which are known to express CAR4; Ref. 20). FACS analysis of BALF eosinophil CAR4 expression confirmed the bone marrow transfer efficiency (Fig. 6A), whereas FACS enumeration revealed no difference in eosinophil levels between mice receiving Car4+/+ and Car4−/− cells (Fig. 6B). For direct evaluation of eosinophil trafficking, we applied a recently reported eosinophil adoptive transfer model (23) and found no migration difference between Car4+/+ and Car4−/− adaptively transferred eosinophils following recipient allergen challenges. Likewise, direct allergen challenge of Car4+/+ and Car4−/− mice (without bone marrow transplantation) revealed no difference in airway eosinophilia (Supplemental Fig. 2C).

The robust CAR4 induction on eosinophils during asthma suggests roles in lung tissue inflammatory processes; however, CAR4 did not directly affect eosinophil lung migration. To further understand the function of eosinophil CAR4 in asthma, we examined the transcriptional change of the whole-lung tissue from Car4+/+ and Car4−/− CD45.2 bone marrow recipient mice that had been subsequently allergen challenged. With asthma induction, we identified 37 genes differentially expressed in the lung of mice that were reconstituted with Car4+/+ compared with Car4−/− hematopoietic cells. The clustered heat-map identified 30 up-regulated genes and 7 downregulated genes (Fig. 6C; see Supplemental Table II for gene list) in Car4+/+ recipients versus Car4−/− recipients (n = 4 mice per group). Gene ontology analysis (http://toppgene.cchmc.org) (24) identified several pulmonary epithelium–related pathways, including keratinization, epithelial differentiation, anion exchange, and epidermal development (the top 10 bio-processes listed in Fig. 6D). The genes and their functional interactions are displayed in a proposed interactive network (Fig. 6E).

Discussion

Recently, a key role of eosinophils in promoting asthma exacerbations has become increasingly recognized, as agents that attenuate eosinophils (e.g., humanized anti–IL-5) are showing promising effects in patients with eosinophilic asthma (6, 25). Responsiveness to biological interventions in asthma, such as anti–IL-5 and anti-IgE, appear to be particularly effective in patients with elevated levels of eosinophils, which are primarily identified by levels of sputum eosinophils (26). These findings, as well as decades of research correlating asthma severity with eosinophil levels and the release of eosinophilic granule constituents into the lung interstitium, highlight the importance of eosinophil activation in asthma. However, specific molecules associated with eosinophil activation have not been convincingly identified, even though these would have great potential value in stratifying patients for therapeutic interventions.

We used genome-wide microarray analysis on sorted murine eosinophils to identify candidate markers of activated eosinophils. The aberrant and robust expression of CAR4 on lung eosinophils was quite surprising, with CAR4 being among the most dysregulated transcripts. There have been no reports regarding hematopoietic expression of this CO2/bicarbonate catalyst gene other than in osteoclasts, which express CAR4 and CAR2 (27). In osteoclasts, CAR2 and CAR4 expression has been speculated to create an acidic milieu. Notably, the human asthmatic airway is an acidic microenvironment (28); therefore, infiltrating leukocytes could potentially modulate the local pH if they expressed surface CAR. Although we did not observe a difference in cellular and intracellular buffering capacity (11, 12) in vitro, we did demonstrate...
the pH buffering capacity of eosinophils, consistent with prior findings (29). CAR4 buffering is also accompanied by a readily detectable intracellular activity (30). As a fine assessment of eosinophil CAR4 function, we also used the lactate-induced acidification assay, in which the subtle cellular change due to extracellular pH buffering is measured. Specifically, CAR4 buffering is mirrored by lactate–H+ cotransport, which depends on CAR4 buffering and results in a reduction in pHi, a known function of CAR4 (21). Despite the negative phenotype, these results do not rule out the possibility that eosinophil-expressed CAR4 contributes to acid balance in the lung in vivo. Of interest, CAR4 is expressed on the outer surface of the plasma membrane of IL-5–activated eosinophils, consistent with a function in trans rather than a role in cis. Nevertheless, CAR4+ and CAR4− eosinophils exhibited similar functional responses in terms of recruitment to the lung and eotaxin signaling.

FIGURE 6. Allergic airway induction in Car4 bone marrow chimeric mice. (A) CD45.1 recipients were transplanted with CD45.2 Car4+/+ and Car4−/− bone marrow after irradiation and were subsequently allergen challenged after ensuring CD11b+ engraftment >99%. Eosinophilia was measured in Car4+/+ and Car4−/− recipients, and BALF eosinophil (EOS) Car4 donor genotype was verified. (B) Total BALF eosinophil levels were quantified by FACS with serial gating (n = 7 per group, mean ± SEM). (C) From the same bone marrow transplant experiment, lung tissue microarray identified a cluster of 37 dysregulated genes (30 upregulated and 7 downregulated, Car4+/+ versus Car4−/− donor), with a statistical criteria of corrected p < 0.01 (Westfall–Young false correction) and fold change > 1.414. N/A, transcripts associated with unknown genes. (D) On the basis of these 37 dysregulated genes, gene ontology analysis identified the top 10 biological processes potentially associated with CAR4 functionality. The p values and number of entities in each category are shown. (E) A schematic illustration of the interactive biological processes/pathways involving these 37 dysregulated genes.
CAR4 UPREGULATED ON EOSINOPHILS BY IL-5 IN ASTHMA

is conceivable that the novel function of CAR4 in asthma pathogenesis is related to the eosi

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