Cationic amino acid transporter 2 regulates inflammatory homeostasis in the lung

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Arginine is an amino acid that serves as a substrate for nitric oxide synthase and arginase. As such, arginine has the potential to influence diverse fundamental processes in the lung. Here we report that the arginine transport protein, cationic amino acid transporter (CAT)2, has a critical role in regulating lung inflammatory responses. Analysis of CAT2-deficient mice revealed spontaneous inflammation in the lung. Marked eosinophilia, associated with up-regulation of eotaxin-1, was present in the bronchoalveolar lavage fluid of 3-week-old CAT2-deficient mice. The eosinophilia was gradually replaced by neutrophilia in adult mice, while eotaxin-1 levels decreased and GRO-α levels increased. Despite the presence of activated alveolar macrophages in CAT2-deficient mice, NO production was compromised in these cells. Examination of dendritic cell activation, which can be affected by NO release, indicated increased dendritic cell activation in the lungs of CAT2-deficient mice. This process was accompanied by an increase in the number of memory T cells. Thus, our data suggest that CAT2 regulates anti-inflammatory processes in the lungs via regulation of dendritic cell activation and subsequent T cell responses.

inflammation | dendritic cells | arginase | nitric oxide

The lungs are at the interface between the host and the environment and are thus constantly exposed to proinflammatory stimuli. Accordingly, mechanisms need to be in place to prevent excessive responses to innocuous stimuli. Any perturbation of the normal balance between inflammatory stimuli and antiinflammatory processes can have profound effects on lung homeostasis, as demonstrated by the lung inflammation seen in mice deficient in the surfactant proteins A or D (1, 2). Alveolar macrophages play a pivotal role in balancing lung inflammation; for example, they normally inhibit immune responses to baseline innocuous antigens but at the same time have a critical role in clearing antigens by phagocytosis (3). Interestingly, mice deficient in granulocyte/macrophage colony-stimulating factor, which is critical for proper development of alveolar macrophages, have inflammation in the lung at baseline (4, 5). Understanding the mechanisms of tolerance to inhaled environmental antigens is relevant to elucidating the cause of increasingly prevalent lung diseases, such as asthma, that involve hypersensitivity to inhaled antigens.

Recent studies have implicated amino acids, specifically tryptophan and arginine, in the regulation of immunity and tolerance. Elegant studies demonstrated an important role for tryptophan metabolism through indoleamine 2,3-dioxygenase (IDO) in inhibition of experimental asthma (6). However, the role of arginine transport and metabolism remains unclear. Among the transport systems that mediate L-arginine uptake, cationic amino acid transporters (CAT1, -2, or -3) are considered to be the major arginine transporters in most cells and tissues (7). Intracellular arginine is metabolized by both the nitric oxide synthase (NOS) and arginase pathways. The product of the former, NO, has been implicated in the regulation of both inflammation and airway tone. Similarly, products of the arginase pathway, such as ornithine, are regulators of key processes involved in lung inflammation, including cell hyperplasia and collagen deposition (8, 9). We chose to focus on CAT2 because of its essential role in arginine transport in immune cells, including peritoneal macrophages (10). Defining the role of arginine and its transport protein CAT2 has been recently aided by the generation of CAT2-deficient mice (10). Although these mice are grossly normal, their peritoneal macrophages have a 95% decrease in L-arginine uptake and a marked impairment in NO production (10, 11). In contrast, fibroblasts are able to compensate for CAT2-deficiency and have only mild impairment in NO production (12). Because arginine entry into the NOS and arginase pathways could have multiple effects on lung inflammation, both positive and negative, we used CAT2-deficient mice to test the net effect of reducing transport of arginine into macrophages, hypothesizing that transport of arginine via CAT2 has an important role in lung inflammation. Our findings demonstrate that CAT2 is an essential part of the host protective immune apparatus in the lung.

Results

Baseline Inflammation in CAT2-Deficient Mice. To study the role of CAT2 in lung inflammation, we examined CAT2-deficient mice. The bronchoalveolar lavage fluid (BALF) demonstrated marked neutrophilia in adult CAT2-deficient mice (Fig. 1*A*, $P < 0.01$). Additionally, in contrast to wild-type lung tissue that was devoid of overt inflammation (Fig. 1*B*), the lungs of CAT2-deficient mice demonstrated perivascular and peribronchial infiltration with mononuclear cells, morphologically most consistent with lymphocytes (Fig. 1*C*). To characterize the infiltrating cells, we performed immunohistochemistry for CD3 and B220. The perivascular/peribronchial infiltrates, present only in CAT2-deficient mice, were composed of both B lymphocytes ($B220^+$) and T lymphocytes ($CD3^+$). The majority ($63.2 \pm 16.6\%$) of the cells were $B220^+$. Furthermore, there was an increase in B cell numbers in the peripheral areas of the lung, not associated with large airways or blood vessels (0.48 ± 0.1 and 3.2 ± 1.6 cells per high power field in wild-type and CAT2-deficient mice, respectively; $n = 3$, $P = 0.01$). Furthermore, focal areas of infiltration with atypical, foamy alveolar macrophages were also observed (data not shown). Similarly, macrophages in the BALF were morphologically activated (enlarged, vacuolated cytoplasm with occasional multinucleated cells; Fig. 1*D*). Although present in mice on both the FVB/N and C57BL/6 background (Fig. 1*A* *Inset*), the inflammation was more pronounced in CAT2-

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Abbreviations: BALF, bronchoalveolar lavage fluid; CAT, cationic amino acid transporter; DC, dendritic cell; MBP, major basic protein; NOS, nitric oxide synthase.

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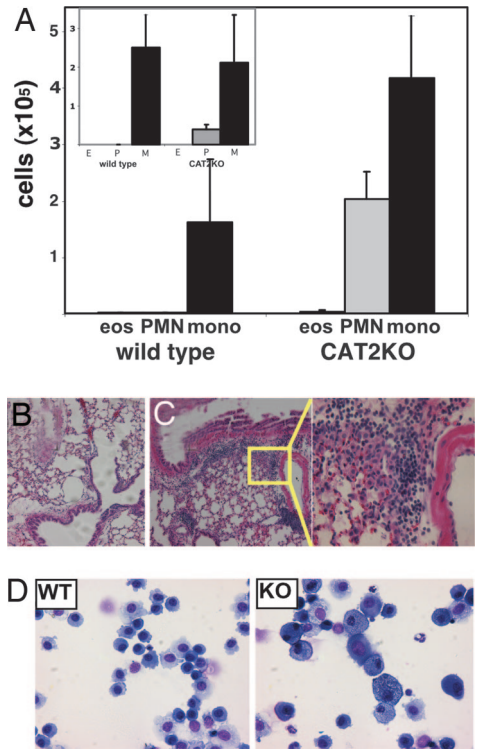

Fig. 1. CAT2$^{-/-}$ mice develop spontaneous lung inflammation. (*A*) BALF was performed on adult (11-week-old) wild-type (FVB/N) and CAT2-deficient (CAT2KO) mice (n = 4–5 mice per group), and cell types were differentiated by morphology (eos, eosinophils; PMN, neutrophils; and mono, mononuclear cells). (*A Inset*) Data from C57BL/6 mice. Lung tissue from a wild-type (*B*) and CAT2-deficient mouse stained with H&E (*C*) shown with higher magnification of the boxed detail. (*D*) Cytospin preparations of BAL cells from wild-type (WT) and CAT2-deficient (KO) mice at 100× magnification are shown.

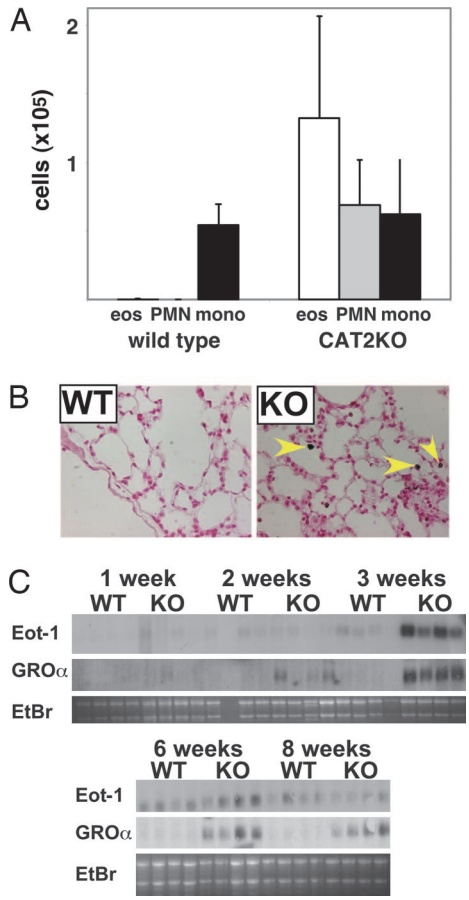

Fig. 2. Inflammation in 3-week-old CAT2-deficient mice. (*A*) BALF was performed on wild-type and CAT2-deficient (CAT2KO) mice (n = 4 and 9 mice, respectively) at 3 weeks of age, and cell types were differentiated by morphology (eos, eosinophils; PMN, neutrophils; and mono, mononuclear cells). (*B*) Tissue sections were stained for eosinophils by using an anti-MBP antibody. Arrowheads point to MBP-positive cells in CAT2-deficient mice. (*C*) Northern blot analysis of lung tissue of CAT2-deficient mice for chemokine expression is shown.

deficient mice in the FVB/N background. Mice of both genders were studied and similarly affected (data not shown). Other organs examined (including jejunum, liver, and kidney) did not display any baseline inflammation (data not shown).

Based on the presence of chronic pulmonary inflammation, we hypothesized that there might be remodeling changes in CAT2-deficient lungs. To assess this possibility, we measured the deposition of collagen, by examining the level of hydroxyproline in the lung. There was a significant increase in the hydroxyproline level in CAT2-deficient mice (data not shown). This increase (20.4 ± 6.2%, average of three experiments) is comparable with that seen in mouse models of allergic airway inflammation (13, 14).

To further characterize the effect of CAT2-deficiency on baseline lung inflammation in CAT2-deficient mice, we performed analysis of inflammatory changes in the lungs of CAT2-deficient mice at different ages. In contrast to what was observed in adult mice (Fig. 1*A*), 3-week-old CAT2-deficient mice had a large number of eosinophils in the alveolar space (Fig. 2*A*, $P < 0.01$). This finding was confirmed by anti-major basic protein (anti-MBP) staining of lung tissue (Fig. 2*B*). Quantitation of anti-MBP staining at 3 weeks of age demonstrated 0.1 ± 0.1 and 2.3 ± 1.2 eosinophils per high power field in wild-type and CAT2-deficient mice, respectively (n = 4 mice per group; $P = 0.01$). Subsequent kinetic analysis demonstrated peak eosinophilia in the alveolar space from 3–4 weeks of age, which was gradually replaced by neutrophilia (Fig. 6, which is published as supporting information on the PNAS web site). By 6 weeks of age there were no eosinophils, but there were large numbers of neutrophils in the alveolar spaces of CAT2-deficient mice, as seen in Fig. 1. Importantly, mice were housed in a specific-pathogen-free facility, in which sentinel mice are routinely examined for evidence of bacterial, viral, and parasitic infections. Furthermore, individual CAT2 mice were found to be free of specific pathogens. Finally, we homogenized lungs from CAT2-deficient and wild-type mice (ages: 3 days, 3 weeks, and adult) and plated serial dilutions on blood agar. We did not find any increased numbers of colonies from CAT2-deficient mice (data not shown). Taken together, infectious agents are not a likely source of the observed chronic inflammation in the lungs of CAT2-deficient mice.

We hypothesized that CAT2-deficient mice might have a temporal dysregulation of chemokine expression in the lung leading to increased inflammatory cell recruitment. To test this hypothesis, we analyzed lung RNA for the expression of chemokines by microarray analysis. In 3-week-old mice, of the 35 chemokines represented on the Affymetrix (Santa Clara, CA) MOE 430 chip, 17 were > 2-fold increased in CAT2-deficient mice (data not shown). Those included chemokines active on eosinophils (eotaxin-1 and -2), neutrophils (CXCL1, CXCL2, CXCL5), monocytes (CCL2, CCL3, CCL4, CCL7, CCL12), T cells (CCL17, CCL22, CXCL9, CXCL10), and B cells (CXCL13). To determine the temporal relationship between chemokine expression and granulocyte (eosinophil versus neutrophil) accumulation, we further analyzed lung RNA for the expression of eosinophil- and neutrophil-tropic chemokines,

eotaxin-1, and GRO-α, respectively, by Northern blot analysis. The lungs of mice at weaning (3 weeks) expressed high levels of eotaxin-1, consistent with the eosinophil accumulation seen at this age (Fig. 2*A*). In contrast, the lungs of adult mice expressed high levels of GRO-α, consistent with the neutrophil accumulation seen at this age (Fig. 2*C* and data not shown); notably, the level of eotaxin-1 was reduced to levels similar to wild-type mice at this time point. Thus, these data show increased expression of eosinophil and neutrophil chemokines in weaning and adult mice, respectively, suggesting that these inflammatory cells accumulate because of chemokine-mediated recruitment from the peripheral blood. Finally, to investigate the source of chemokine expression, we performed *in situ* hybridization for eotaxin-1 in the lungs of 3-week-old wild-type and CAT2-deficient mice. There was no detectable expression of eotaxin-1 in the lungs of wild-type mice (data not shown). As seen in Fig. 7, which is published as supporting information on the PNAS web site, eotaxin-1 was expressed in mononuclear inflammatory cells in CAT2-deficient mice. No expression was observed in epithelial cells (Fig. 7 and data not shown). Given the expression of eotaxin chemokines in the lungs, we hypothesized that there is a Th2-deviated milieu in 3-week-old CAT2-deficient mice. To test this hypothesis, we measured the level of IL-4 in the plasma of 3-week-old CAT2-deficient mice by using cytokine capture assay. These analyses demonstrated an increase in IL-4 levels (from 62.1 ± 1.6 to 103.2 ± 20.1 in wild-type and CAT2-deficient mice, respectively, representative of two experiments with three mice per group, $P = 0.02$). This finding was specific to mice at weaning as in adult CAT2-deficient mice the level of IL-4 was comparable with that of wild-type mice (44.7 ± 8.5 and 58.3 ± 15.9 in wild-type and CAT2-deficient mice, respectively, $P = 0.26$).

Lung Macrophages Are Activated in CAT2-Deficient Mice. Previous studies have shown that alveolar macrophages have the capacity to suppress inflammatory reactions to innocuous antigens in the lung (15). Based on these studies, and our finding of morphologically activated macrophages in the BALF of CAT2-deficient mice, we analyzed the MHC class II expression on $CD11c^+$ high autofluorescent cells in enzymatically digested lungs [consistent with macrophages (16)]. The percentage of high autofluorescent cells that express MHC class II increased from 39.2 ± 4.3 to 69.0 ± 1.8 in wild-type and CAT2-deficient mice ($n = 3, P < 0.001$), respectively.

CAT2 Is Required for NOS Activity in Alveolar Macrophages. Previous studies have demonstrated that peritoneal and bone marrow-derived macrophages require CAT2 for NOS activity (10, 17). However, other cell types (i.e., fibroblasts) are able to produce NO even in the absence of CAT2 (12). Because alveolar macrophages differ from monocytes and macrophages from other locations in many aspects (18, 19) and because NO was suggested as a potential mechanism of action for alveolar macrophage-mediated suppression of lung inflammation (15), we tested the hypothesis that CAT2 was required for NOS activity in alveolar macrophages. Alveolar macrophages were collected by bronchoalveolar lavage of wild-type and CAT2-deficient mice, and activated cell supernatants were assessed for NO_2^- production. As shown in Fig. 3, there was a 81.2% (from 0.8 ± 0.01 to 0.1 ± 0.04, $n = 2$–3, $P < 0.01$) decrease in NOS activity in CAT2-deficient alveolar macrophages. As a control, there was also a 97.6% decrease in NOS activity in peritoneal macrophages. These data demonstrate that CAT2 is required for NOS activity in alveolar macrophages and that alveolar macrophages are functionally impaired in CAT2-deficient mice despite their activated phenotype.

Dendritic Cell Subsets and Activation in the Lungs of CAT2-Deficient Mice. Previous studies have suggested that alveolar macrophages suppress inflammatory responses to innocuous antigens in the lung by inhibiting dendritic cell activation via NO (15). To begin testing the hypothesis that dendritic cells (DC) are involved in the phenotype of CAT2-deficient mice, we aimed to characterize the subpopulations, numbers, and activation status of DCs. Accordingly, we performed flow cytometry for CD11c, CD11b, Gr-1, and MHC class II on enzymatically digested lungs from CAT2-deficient and wild-type mice (Fig. 4*A*). Gating strategy from a representative mouse is shown in Fig. 8, which is published as supporting information on the PNAS web site. Briefly, $CD11c^+$ cells were further differentiated into $CD11b^+Gr1^-$ (most consistent with myeloid DC, mDC); $CD11b^-Gr1^-$ (most consistent with lymphoid DC, lDC and/or lung macrophages, see below) and $CD11b^-Gr1^+$ (most consistent with plasmacytoid DC, pDC) and $CD11b^+Gr1^+$ [undefined population consistent with granulocytes, myeloid suppressor cells (20), and pDCs differentiating to mDC during viral infection (21)]. We have previously demonstrated the appropriate function of DC after our gating strategy (22, 23). There was a significant increase in the proportion and total number of $CD11b^+Gr\text{-}1^-$ DC compared with wild-type mice (Fig. 4*B*). For instance, the percentage of $CD11b^+Gr\text{-}1^-$ DC (among all $CD11c^+$, vital dye [7-AAD]-negative cells) was 29.7 ± 1.6 and 43.9 ± 6.4% in wild-type and CAT2-deficient mice, respectively ($n = 3, P = 0.02$). Similarly, the number of lung $CD11b^+Gr\text{-}1^-$ DC increased from $1.4 \pm 0.4 \times 10^5$ in wild-type mice to $3.8 \pm 0.7 \times 10^5$ in CAT2-deficient mice ($P = 0.009$). The total number of $CD11b^-Gr1^-$ DC or $CD11b^-Gr1^+$ DC did not change.

To test the hypothesis that DCs are activated in CAT2-deficient mice, we assessed their expression of MHC class II. There was a significant increase in the level of MHC class II expression on DCs in the lung; this increase was most notable on $CD11b^-Gr1^-$ DC (Fig. 4*B*). For instance, the percentage of MHC class II positive $CD11b^-Gr1^-$ DC increased from 58.7 ± 3.8% in wild-type mice to 78.4 ± 4.6% in CAT2-deficient mice ($n = 3, P = 0.004$). Similarly, the mean channel fluorescence increased from 800 ± 144 to 1961.3 ± 370 in wild-type and CAT2-deficient mice, respectively ($n = 3, P = 0.007$).

Lung macrophages are often indistinguishable from DCs by flow cytometry because they express CD11c but not (or low levels of) F4/80 or other common macrophage markers (16, 24). Thus, it remained possible that macrophages were present in the $CD11b^-Gr1^-$ DC compartment. Recently, Vermaelen *et al.* (16) demonstrated that lung macrophages can be distinguished from DCs based on their high autofluorescence in the FL1 channel. Indeed, this independent analysis confirmed that the number of

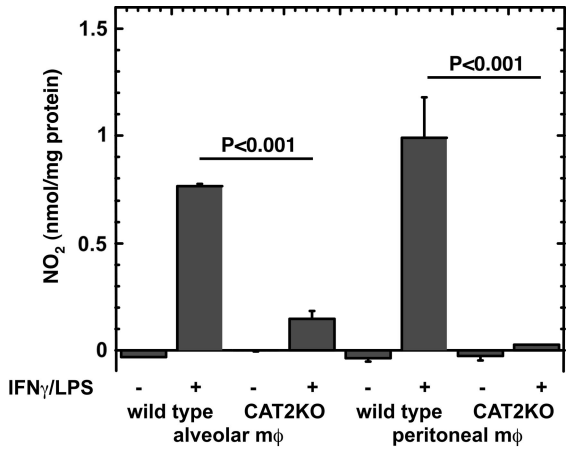

Fig. 3. Role of CAT2 on alveolar macrophages. Alveolar macrophages from wild-type (WT) and CAT2-deficient (CAT2KO) mice were collected and stimulated with IFN-γ and LPS. NO_2^- in the supernatant was measured and normalized to protein content of monolayer cells.

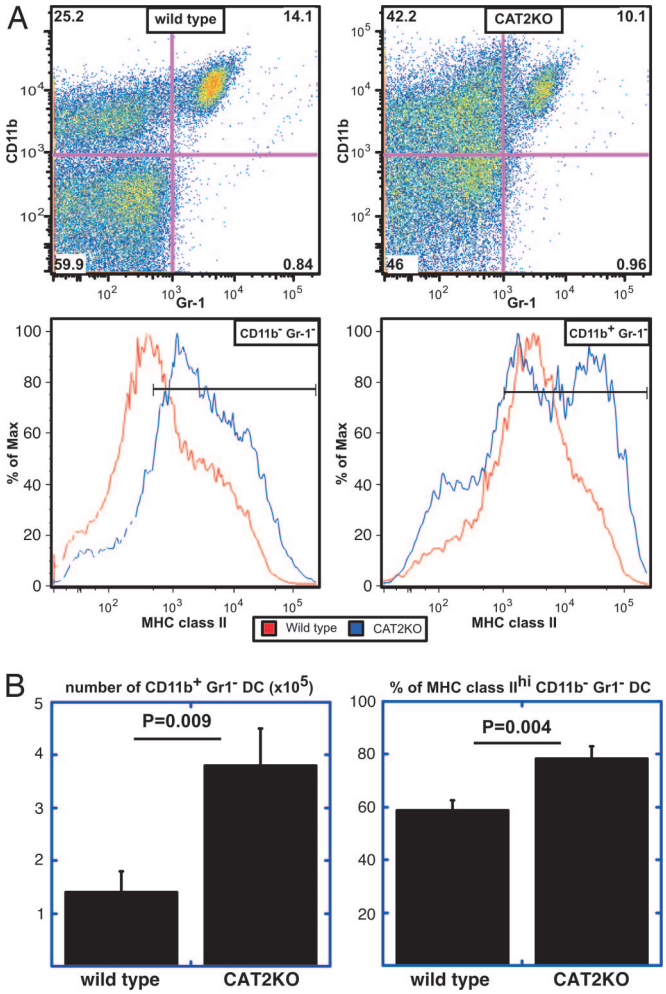

Fig. 4. Dendritic cell phenotype in CAT2-deficient mice. (*A*) Lung cells were stained with antibodies against CD11c, CD11b, Gr-1, and MHC class II. The 7-AAD was used to differentiate live cells. Flow cytometry was performed: The 7-AAD$^-$ CD11c$^+$ cells were differentiated based on CD11b and Gr-1 expression. Finally, to assess activation level, MHC class II expression of individual subpopulation is shown in histograms. (*B*) The number of CD11b$^+$Gr1$^-$ DC (*Left*) and percentage of MHC class II high CD11b$^-$Gr1$^-$ DC (*Right*) in lungs of wild-type and CAT2-deficient (CAT2KO) mice is shown. Representative experiment from three to five experiments is shown.

myeloid cells is increased and that DCs are activated in the lungs of CAT2-deficient mice (data not shown).

Finally, we hypothesized DC activation would occur with the early inflammatory phenotype. Similar to what was observed in adult mice, the number of CD11b$^+$Gr1$^-$ DC increased from 3.8 ± 1.9 to $8.1 \pm 0.6 \times 10^5$ ($P = 0.02$, $n = 3$) in CAT2-deficient mice that were 2–3 weeks old. Furthermore, the percentage of CD11b$^-$Gr1$^-$ DC that express high levels of MHC class II increased from 54.6 ± 10.9 to 72.1 ± 3.7 ($P = 0.05$, $n = 3$).

Activated/Memory Phenotype of T Cells in CAT2-Deficient Mice. The observed chronic inflammation and increased DC activation in CAT2-deficient mice suggested increased adaptive immune responses. We hypothesized that CAT2-deficient mice would have an increased proportion of activated and memory T cells. To test this hypothesis, we assessed the proportion of CD44^{hi} cells in TCRβ^+ CD4$^+$ cells in the lung of wild-type and CAT2-deficient mice. The proportion of CD44^{hi} cells was increased 2.4 ± 0.4 fold (mean of three experiments) in CAT2-deficient mice (representative experiment shown in Fig. 5), suggesting an increase in T helper activated

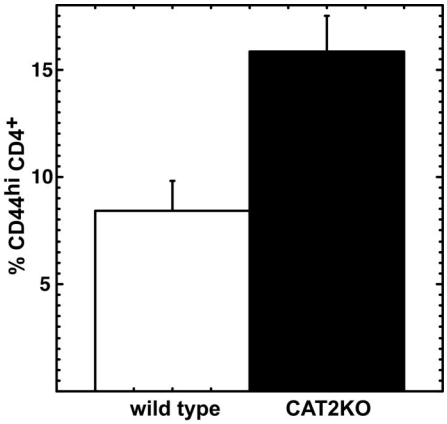

Fig. 5. Memory phenotype of T cells in the lungs of CAT2-deficient mice. Memory cells were determined by flow cytometry for CD4, CD44, and TCRβ in wild-type (WT) and CAT2-deficient (CAT2KO) mice in the collagenase-dispersed lung. Data are from a representative experiment (of three) performed with three to four mice in each group ($P < 0.01$).

and memory cells. This increase was already evident in 2- to 3-week-old mice, which had 11.9 ± 0.7 and $31 \pm 3.8\%$ CD44^{hi} cells in TCRβ^+ CD4$^+$ cells in the lung of wild-type and CAT2-deficient mice, respectively ($n = 3$, $P = 0.02$). Similar results were obtained when memory cells were defined as CD62L$^{lo/neg}$ cells (data not shown).

Discussion

We have demonstrated that CAT2-deficient mice exhibit baseline inflammation in the lungs in the absence of identifiable pathogenic stimuli. These findings have led us to the hypothesis that CAT2 is involved in the suppression of inflammation in the lungs. Based on our data and previous publications, we propose the following mechanism: In wild-type lungs, arginine metabolism via NOS in alveolar macrophages leads to suppression of DC activation in response to innocuous antigens (15). However, when CAT2 is absent, the impaired NOS activity in alveolar macrophages leads to unopposed DC activation and function. To support our model, we demonstrate that NO production in alveolar macrophages depends on CAT2. Importantly, previous studies (10) have demonstrated that there was no difference in the expression of NOS2, CAT1, and CAT3 in multiple tissues including the lung. Furthermore, we demonstrate that DC are activated in CAT2-deficient mice. This finding correlates with the observed baseline lung inflammation and immunopathology, including granulocytosis (in response to chemokine expression), lymphoid hyperplasia (involving increased memory T cells), and structural changes (i.e., collagen deposition). The presence of the specific baseline disease in the lungs may be secondary to the large surface area of the lung that promotes exposure to higher oxygen concentrations, environmental irritants, pollutants, and infectious agents. These results have clinical significance and broad implications for the numerous poorly understood lung diseases involving aberrant lung inflammation and remodeling (e.g., asthma, idiopathic pulmonary fibrosis and COPD). In particular, our results show that alterations in the function and/or availability of CAT2 and/or its substrate arginine may be a contributing factor to inflammatory lung diseases.

Alveolar macrophages have a critical role in balancing lung inflammation; for example, they normally inhibit immune responses to baseline innocuous antigens but at the same time have a critical role in clearing antigens by phagocytosis (3). Specifically, the resident alveolar macrophage population actively suppresses MHC class II expression and the antigen presenting function of lung DC *in situ*, as well as in coculture systems (15). Importantly, the NO

synthase function in alveolar macrophages has been implicated in this process (15). For instance, the inhibitory effect of alveolar macrophages on the antigen-presenting cell function of DC is abrogated by a NOS inhibitor *in vitro*. As an example of lung DC hyporesponsiveness, it has been demonstrated that recently isolated ovalbumin-pulsed lung DCs stimulate weak Th2 responses compared with *in vitro* cultured or granulocyte/macrophage colony-stimulating factor-treated DC that stimulate strong Th2 responses (25). It seems likely that CAT2 and its subsequent associate products, NO and ornithine, may be involved in regulating DCs. We speculate that the lack of CAT2, with corresponding decrease in NO production by macrophages, is a potential mechanism for the observed baseline inflammation. In support of this model, previous studies have shown alveolar macrophages and dendritic cells in close proximity to each other (15). Alternatively, CAT2 may affect arginase activity, which has been demonstrated as a mechanism involved in macrophage-mediated T cell suppression (26). The effect of arginase activity in macrophage-mediated DC suppression has not yet been studied.

The eosinophil infiltration observed in CAT2-deficient mice at 3 weeks of age, and subsequent neutrophil and lymphocyte infiltration, may be markers of heightened Th2 and Th1 immunity, respectively. Indeed, it has been demonstrated that DC from the respiratory tract preferentially initiate Th2 immunity (27). However, when activated, they shift to Th1 immunity (27). Because DC populate the lung and become fully mature after weaning (≈3 weeks of age) (28), we propose that this maturation correlates with the switch from eosinophilia to neutrophilia in CAT2-deficient mice.

It remains possible that the observed phenotype in CAT2-deficient mice is because of defects in other cell types. Bone marrow chimeras and adoptive transfer studies, may be useful to determine the requirement for macrophages for the observed phenotype. Indeed, our initial experiments revealed a 6.5-fold increase in BALF granulocytes when knockout bone marrow was transferred, compared with wild-type bone marrow, into lethally irradiated knockout mice (data not shown). It also remains possible that lymphocytes accumulate because of an intrinsic defect of CAT2 and NOS activity in these cells. For instance, it has recently been demonstrated that inducible NOS-deficient mice have an increased pool of memory T cells because of dysregulation of death proteins (29). We favor the hypothesis that the main defect is within the macrophage or DC lineage because those cells have been demonstrated to be the main orchestrators of both innate and adaptive immune responses (15, 27). Furthermore, our recent preliminary data demonstrate that the $CD4^+$ $CD44^{hi}$ $CD62L^{lo/neg}$ cells in the lungs of CAT2-deficient mice are CD69-positive, and that there is an increase in CD25-positive $CD4^+$ cells [consistent with recently activated and proliferating cells (data not shown)]. Accumulation of memory cells could be because of increased generation or decreased death; data showing increased activation and proliferation markers suggest that increased generation of memory cells is more likely the main mechanism for memory cell accumulation. It is important to note that there have not been reports of baseline inflammation in the lungs of single NOS isoform-deficient mice. We believe that the deficiency of a single isoform of either NOS or arginase may not be sufficient for the phenotype, whereas the deficiency of CAT2, which becomes a functional deficiency of multiple isoforms of both the NOS and arginase pathway, does not allow for redundancy, and thus the phenotype manifests itself. This theory is supported by the *in vitro* finding that in the myeloid suppressor cell system, both pathways need to be inhibited in myeloid suppressor cells to release T cell proliferation (26). However, it still remains possible that the lung phenotype observed in CAT2-deficient mice occurs independently of NOS and/or arginase. Consistent with this possibility, CAT2 also serves as a transporter of lysine and ornithine. We have chosen to focus on arginine because of its role in inflammation and immunity. It is interesting to draw parallels between the arginine metabolism and tryptophan metabolism via indoleamine 2,3-dioxygenase (IDO) in macrophages and DCs; products of the latter pathway have been shown to fundamentally regulate development of tolerance versus immunity. A subset of human antigen-presenting cells that expresses IDO has been shown to maintain T cell homeostasis and self-tolerance through inhibition of T cell proliferation (30).

In summary, we report that CAT2 deficiency leads to baseline inflammation in the lung. Furthermore, we propose a model in which CAT2 expression on alveolar macrophages is required for NO production and the subsequent suppression of DC activation. This mechanism maintains inflammatory homeostasis in the lungs despite constant exposure to potentially inflammatory triggers. These results establish a mechanism by which macrophages, via CAT2, promote tolerance and inflammatory homeostasis in the lung. Thus, we have identified a potential pathway that may be contributing to the reduction in tolerance observed in several lung inflammatory diseases.

Materials and Methods

Mice. CAT2-deficient mice were described (10). Mice were labeled as adult if at least 8 weeks old.

Northern Blot Analysis. RNA was electrophoresed, transferred, and cross-linked as reported in refs. 31–33. The cDNA probes were obtained from commercially available vectors (Image Consortium, Livermore, CA), or amplified by RT-PCR, sequence confirmed, and radiolabeled with ^{32}P, and hybridized by using standard conditions.

Hydroxyproline Measurement. Collagen content of lungs was measured as hydroxyproline by the technique of Bergman and Loxley (34) after hydrolysis in 6 M HCl at 110°C for 18 h.

Immunohistochemistry. Eosinophils in the tissue were differentially stained by using a rabbit anti-MBP antibody (a kind gift from J. and N. Lee, Mayo Clinic, Scottsdale, AZ) as described in ref. 35. B cells were stained by anti-B220 and T cells by anti-CD3 (BD Pharmingen, Franklin Lakes, NJ), by using standard techniques.

Flow Cytometry. Lungs were enzymatically digested with Liberase CI enzyme blend (0.5 mg/ml; Roche, Basel, Switzerland) and DNase (0.5 mg/ml; Sigma, St. Louis, MO) at 37°C for 30 min. Cells were blocked with Fc block, and stained with the following antibodies: anti-CD11b-PE Cy7, CD11c-APC, Gr1-APC Cy7, and MHC class II-PE (BD PharMingen). After two washes, labeled cells were resuspended in vital dye 7-AAD and subjected to flow-cytometry on FACSvantage flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed by using FlowJo software (Tree-Star, Ashland, OR). In some experiments, autofluorescence of macrophages in FL-1 was used to exclude macrophages from CD11c-positive cells, as described (16). T cell staining was performed by using anti-CD4-PE Cy5, CD8-APC, CD44-FITC, and TCRβ-PE (BD PharMingen).

NO Production. Alveolar macrophages were collected by BAL from wild-type and CAT2-deficient mice. Cells were allowed to adhere for 2 h, and nonadherent cells were removed. Cells were then stimulated with IFN-γ (20 ng/ml) and LPS (100 ng/ml) for ≈24 h. Supernatant NO_2^- was measured by using the Griess reagent.

Microarray Analysis. Microarray analysis was performed essentially as described in refs. 36 and 37. Briefly, whole lung RNA from 3-week-old wild-type and CAT2-deficient mice was isolated by using TRIzol reagent. RNA was hybridized to the MOE430 Ge-

neChip (Affymetrix), by using one mouse per chip. All chemokines were filtered, and those with a fold difference >2 with a $P < 0.05$ were further analyzed.

***In Situ* Hybridization of Mouse Lung.** *In situ* hybridization was performed as described (36, 38). In brief, murine eotaxin-1 cDNA sense and anti-sense probes were generated by T7 and T3 RNA polymerase, respectively. The ^{35}S-labeled probes were hybridized and washed under high stringency conditions.

***In Vivo* Cytokine Capture Assay (IVCCA).** *In vivo* IL-4 production was analyzed with the IVCCA, which increases the sensitivity of cytokine detection 100-fold, as described (39).

Statistical Analysis. The significance of differences between the means of experimental groups was analyzed by using Student's unpaired *t* test. Values are reported as the mean ± standard deviation.

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