Inflammatory macrophage memory in nonsteroidal anti-inflammatory drug–exacerbated respiratory disease

Pascal Haimerl, MSc,a Ulrike Bernhardt,a,b Sonja Schindela,a Fiona D. R. Henkel, MSc,a Antonie Lechner, RPh,a Ulrich M. Zissler, PhD,a Xavier Pastor, MSc,a Dominique Thomas, PhD,d Alexander Cecil, PhD,e Yan Ge, PhD,f Mark Haid, Dipl Biol,a Cornelia Prehn, PhD,a Janina Tokarz, PhD,a,d Matthias Heinig, PhD,a Jerzy Adamski, PhD,e,h,i Carsten B. Schmidt-Weber, PhD,a,* Adam M. Chaker, MD,a,b and Julia Esser-von Bieren, PhDa Munich, Neuherberg, Frankfurt, Dresden, and Freising-Weißenstephan, Germany, and Singapore

GRAPHICAL ABSTRACT

Background: Nonsteroidal anti-inflammatory drug–exacerbated respiratory disease (N-ERD) is a chronic inflammatory condition, which is driven by an aberrant arachidonic acid metabolism. Macrophages are major producers of arachidonic acid metabolites and subject to metabolic reprogramming, but they have been neglected in N-ERD.

Objective: This study sought to elucidate a potential metabolic and epigenetic macrophage reprogramming in N-ERD.

Methods: Transcriptional, metabolic, and lipid mediator profiles in macrophages from patients with N-ERD and healthy controls were assessed by RNA sequencing, Seahorse assays, and LC-MS/MS. Metabolites in nasal lining fluid, sputum, and plasma from patients with N-ERD (n = 15) and healthy individuals (n = 10) were quantified by targeted metabolomics analyses. Genome-wide methylomics were deployed to define epigenetic mechanisms of macrophage reprogramming in N-ERD.

From the Center of Allergy and Environment, Technical University of Munich and Helmholtz Zentrum München, Munich; the Department of Otolaryngology, Allergy Section, Klinikum Rechts der Isar, Technical University of Munich; the Institute of Computational Biology, and the Research Unit of Molecular Endocrinology and Metabolism, Genome Analysis Center, Helmholtz Zentrum München, Neuherberg; the Pharmazentrum Frankfurt/Zentrum für Arzneimittel-Forschung, -Entwicklung und -Sicherheit (ZAFES), Institute of Clinical Pharmacology, Goethe University Frankfurt; the Biotechnology Center Dresden, Technical University of Dresden; the German Center for Diabetes Research, Neuherberg; the Lehrstuhl für Experimentelle Genetik, Technische Universität München, Freising-Weißenstephan; and the Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore.

*Member of the German Center of Lung Research.

This study was supported by the Else Kröner-Fresenius-Stiftung (grant 2015_A195), the German Research Foundation (FOR2599, ES 471/3-1), the Fritz Thyssen Stiftung (grant Az. 10.17.2.017MN), and a Helmholtz Young Investigator grant (VI-NG-1331) to JEvB. CSW receives grant support by the German Center for Lung Research (82DZL00302). This study was supported in part by a grant from the German Federal Ministry of Education and Research to the German Center Diabetes Research. Disclosure of potential conflict of interest: C. B. Schmidt-Weber received grant support from Allergopharma, PLS Design, as well as Zeller AG; and received speaker honoraria from Allergopharma. The rest of the authors declare that they have no relevant conflicts of interest. Received for publication February 13, 2020; revised April 4, 2020; accepted for publication April 22, 2020.

Corresponding author: Julia Esser-von Bieren, PhD, Center of Allergy and Environment, Technische Universität München and Helmholtz Zentrum München, 80802 Munich, Germany. E-mail: Julia.esser-von-bieren@tum.de.

© 2020 American Academy of Allergy, Asthma & Immunology https://doi.org/10.1016/j.jaci.2020.04.064
Results: This study shows that N-ERD monocytes/macrophages exhibit an overall reduction in DNA methylation, aberrant metabolic profiles, and an increased expression of chemokines, indicative of a persistent proinflammatory activation. Differentially methylated regions in N-ERD macrophages included genes involved in chemokine signaling and acylcarnitine metabolism. Acylcarnitines were increased in macrophages, sputum, nasal lining fluid, and plasma of patients with N-ERD. On inflammatory challenge, N-ERD macrophages produced increased levels of acylcarnitines, proinflammatory arachidonic acid metabolites, cytokines, and chemokines as compared to healthy macrophages.

Conclusions: Together, these findings decipher a proinflammatory metabolic and epigenetic reprogramming of macrophages in N-ERD. (J Allergy Clin Immunol 2020;nnn;nnn-nnn.)

Key words: Acylcarnitines, chemokines, eicosanoids, lipid mediator, macrophages, metabolomics, nasal polypos, NSAID-exacerbated respiratory disease, trained immunity, type 2 inflammation

Chronic rhinosinusitis with nasal polypos (CRSwNP) affects 0.5% to 4% of the population in most Westernized countries and represents a debilitating disease, which is often refractory to treatment.1 Patients affected by CRSwNP suffer from a significantly reduced quality of life due to nasal obstruction, facial pain, hyposmia, sleep apnea, and increased susceptibility to sinus infections. Nonsteroidal anti-inflammatory drug (NSAID)–exacerbated respiratory disease (N-ERD), the recently chosen term for aspirin-exacerbated respiratory disease and Samter triad,2 represents a particularly severe endotype of CRSwNP, which affects around 10% to 16% of patients with CRSwNP.3,4 N-ERD is characterized by severe and refractory nasal polypsis, bronchial asthma, and intolerance to NSAIDs.5 To date, the pathogenesis of N-ERD remains incompletely understood and curative treatments are lacking.

Multiple studies have implicated a dysregulation of the arachidonic acid (AA) metabolism as well as aberrant type 2 cytokine responses in the pathogenesis of N-ERD.5,7 The aberrant production and signaling of AA metabolites (eicosanoids), in particular leukotrienes (LTs) and prostaglandins (PGs), are hallmarks of N-ERD and determine chronic airway inflammation as well as NSAID-triggered respiratory reactions.8-11 Patients with N-ERD exhibit an overproduction of proinflammatory LTs and PGD2, but a decreased synthesis of the airway-protective cyclooxygenase metabolite PGE2.10 These N-ERD-typic changes in the AA metabolism can be observed in local samples (sputum, saliva, nasal lining/lavage fluid) as well as systemically.12-16 In addition to dysregulation of the AA metabolism, a high body mass index (BMI) and changes in the metabolism of sphingolipids have recently been associated with N-ERD.15,14 This suggested that metabolic aberrations beyond an altered AA metabolism contribute to the pathogenesis of N-ERD.

Macrophages are major cellular players in metabolic reprogramming13,16 and represent an important source and cellular target of fatty acid metabolites during type 2 inflammation.16,19 Despite the abundance of profibrotic macrophages in the nasal mucosa of patients with NSAID-tolerant (NT) CRSwNP and those with N-ERD,20,21 the phenotypes and functions of macrophages in N-ERD are largely unknown. Indeed, patients with N-ERD have a high risk of asthma exacerbations, which has been linked to defective macrophage function.22,23 Macrophage reprogramming has recently been suggested to occur during type 2 airway inflammation, but the underlying mechanisms and functional consequences remain obscure.22

Using global transcriptomics, genome-wide methylomics, and targeted metabolomics, we identify changes in the acylcarnitine metabolism and activation of macrophages as potential drivers of type 2 inflammation in N-ERD. Patients with N-ERD also exhibited elevated acylcarnitine levels in macrophages, nasal lining fluid, sputum, and plasma, and N-ERD macrophages responded to inflammatory challenge with an exaggerated induction of proinflammatory lipid mediators, acylcarnitines, chemokines, and cytokines. The persistent proinflammatory reprogramming of macrophages may contribute to the chronic and therapy-refractory airway inflammation in N-ERD.

METHODS

Materials

A detailed list of all materials used in this study can be found in this article’s Online Repository available at www.jacionline.org.

Patient characterization

Patients with N-ERD and healthy controls were recruited and classified according to their clinical characteristics at the ear, nose, and throat clinic of the Klinikum rechts der Isar (Munich, Germany) (see Table E1 in this article’s Online Repository at www.jacionline.org for an overview). Malm score25 was determined by nasal endoscopy. Healthy controls had no history of chronic rhinosinusitis, nasal polyposis, asthma, or intolerance to NSAIDs. NT controls with CRSwNP had taken NSAIDs within the previous 6 months without any adverse reaction. N-ERD was diagnosed based on physician-diagnosed chronic asthma, CRSwNP, and a history of respiratory reactions to oral NSAIDs. NSAID intolerance in one of the patients had previously been confirmed by aspirin provocation.26 Exclusion criteria were acute airway infections; other systemic immune disorders; pregnancy; cancer; and use of antibiotics, anti-5-lipoxygenase (5-LOX, zileuton), biologics (omalizumab, benralizumab, reslizumab, mepolizumab, dupilumab), and/or oral corticosteroids 4 weeks prior to the study.
Collection of nasal lining fluid
Nasal lining fluid from patients with N-ERD and healthy individuals of cohort 1 were obtained as described previously 27-29 and subjected to metabolomics analyses. For details, see this article’s Online Repository.

Plasma collection and generation of MDMs
Plasma was collected and CD14<sup>+</sup> monocytes were isolated and differentiated into macrophages with features of alveolar monocyte-derived macrophages (aMDMs) as described previously. 19 aMDM supernatants were harvested after 24 hours of culture and used for multiplex cytokine/chemokine and lipid mediator analyses. For details, see this article’s Online Repository.

Sputum induction and isolation of sputum macrophages
Sputum induction and induced sputum-isolated macrophage (sMac) isolation were performed as previously described. 30,31 Sputum samples were subjected to metabolomics and lipid mediator analysis, and sMac were used for RNA sequencing (RNAseq) analysis. For more details, see this article’s Online Repository.

Transcriptomics
Total RNA was extracted and subjected to RNAseq as previously described. 19 For details, see this article’s Online Repository.

Genome-wide methylationomics
Genomic DNA was extracted from CD14<sup>+</sup> monocytes or aMDMs and subjected to whole-genome methylation analysis. For more details, see this article’s Online Repository.

Lipid mediator analysis and metabolomics
Lipid mediator analysis was performed by LC-MS/MS as described previously. 19 A complete list of all measured analytes can be found in an extra Excel file (Microsoft, Redmond, Wash) (see Table E2 in this article’s Online Repository at www.jacionline.org). For a detailed description of targeted lipid mediator and metabolomics analyses, see this article’s Online Repository.

Data analysis
RNAseq differentially expressed gene (DEG) analysis was performed with R (v3.5.0) 33 and DESeq2. 34 Methylation data were analyzed with the Epigenie 35 utilizing limma 36 and DMRcate. 37 ToppGene suite 38 was used for pathway analysis and enrichment. For a detailed description of R packages, see this article’s Online Repository.

Data availability
Sequencing data have been deposited in the ArrayExpress database at European Molecular Biology Laboratory–European Bioinformatics Institute (https://www.ebi.ac.uk/arrayexpress) under accession numbers E-MTAB-7965 (RNAseq) and E-MTAB-8065 (methylation).

Study approval
This study was approved by the local ethics committee at the Klinikum rechts der Isar, Technical University of Munich (internal reference: 422/16).

Written informed consent in accordance with the Declaration of Helsinki was obtained from all patients.

RESULTS
Patients with N-ERD exhibit a persistent proinflammatory macrophage activation
MDMs populate the airways, plasticly respond to inflammatory stimuli, and govern immune responses during type 2 inflammation. 40,41 To identify a potential macrophage reprogramming in N-ERD, we performed an RNAseq analysis of aMDMs, differentiated from CD14<sup>+</sup> blood monocytes from patients with N-ERD and healthy individuals in a lung-adapted cytokine milieu (TGF-β1 and GM-CSF). 19 Despite 1 week of in vitro differentiation, 86 downregulated and 19 upregulated genes were identified in N-ERD as compared to healthy aMDMs (Fig 1, A; see Table E3, A and C, in this article’s Online Repository at www.jacionline.org). This included genes involved in chemotaxis (CXCL1–CXCL3, PPBP, CXCL8, CCL18, CCL20) and host defense (CD1A–CD1C, CLEC10A, CLEC18B) as revealed by functional and pathway analysis (Fig 1, A–C, Table E3, B), suggesting that N-ERD is associated with a persistent proinflammatory activation of monocytes/macrophages.

To further investigate whether proinflammatory macrophages were present in the airways of patients with N-ERD, sMacs of patients with N-ERD were used for transcriptome analysis. To obtain insights into local versus systemic macrophage activation profiles, we compared sputum-derived and blood-monocyte-derived macrophages from the same patients with N-ERD. RNAseq revealed a total of 984 genes to be downregulated and 1869 genes to be upregulated in sMacs as compared to aMDMs (Fig 1, D, Table E3, D and F). Most DEGs were associated with host defense and immune regulation as revealed by enrichment and pathway analysis (Fig 1, E, Table E3, E). sMacs from patients with N-ERD exhibited high expression of genes involved in chemotaxis (CXCL1–CXCL3, CXCL8, CXCL9, CCL18, CCL20), immune regulation (IDO1, IL10, IL23A, IL27, SCGB1A1), T-cell priming (HLA, CD28, CD40, CD80), and host defense (TLR2, TLR5, TLR10, CD1A–CD1C), suggestive of a more activated profile in comparison to aMDMs (Fig 1, F, Table E3, D and F). We also found a distinct eicosanoid gene expression signature in aMDMs (ALOX5AP, CYSLTR1, LT4H4, PTGS1, PTGD5) versus sMacs (ALOX15, CYSLTR2, PTGS2, HPGDS, PTGER2–PTGER3, PTGES), which was indicative of a LT-dominated eicosanoid profile in aMDMs but indicative of a 15-LOX/COX–dominated profile in sMacs (Fig 1, F). Although low numbers of sMacs recovered from healthy individuals precluded a comparison of N-ERD sMacs to healthy sMacs, this suggested that monocytes and macrophages with a persistent proinflammatory phenotype are present in the blood and airways of patients with N-ERD.
FIG 1. Macrophages from patients with N-ERD exhibit proinflammatory gene expression profiles. A-C, Transcriptome analysis of N-ERD (n = 5) and healthy (n = 4) aMDMs. A, Volcano plot of DEGs; significant changes in blue (decrease) or red (increase); thresholds were set to $P_{adj} < 0.05$ and log$_2$-fold change $> 11/21$. B, Top 10 significant pathways obtained by ToppGene analysis. See Table E3, B, for the complete list. D to F, Transcriptome analysis of aMDMs and sMacs from N-ERD (n = 5). D, Volcano plot of DEGs; significant changes shown in blue (decrease) or red (increase); thresholds were set to $P_{adj} < 0.01$ and log$_2$-fold change $> 11/21$. E, Top 13 significant pathways obtained by ToppGene analysis. See Table E3, E, for the complete list. Heat maps of selected DEGs (Z score) between patients with N-ERD and healthy individuals (C) or cell types (F). ECM, Extracellular matrix.
Repository at www.jacionline.org). As a dysregulated polyunsaturated fatty acid (PUFA) metabolism is a hallmark of N-ERD, we further investigated whether PUFA metabolites were altered in sputum, nasal lining fluid, or macrophages of our N-ERD cohort. Indeed, we confirmed a tendency of higher LT but lower PGE2 production in the airways of patients with N-ERD by LC-MS/MS (see Fig E1 and Table E5, A and B, in this article’s Online Repository at www.jacionline.org). To study whether aMDMs from patients with N-ERD exhibit an altered capacity to produce eicosanoids, we assessed levels of PUFA metabolites by LC-MS/MS after Ca^{2+} ionophore (A23187) stimulation. Significantly more AA-derived 5-LOX metabolites (5-hydroxyeicosapentaenoic acid/5-hydroxyeicosatetraenoic acid/5-oxo-eicosatetraenoic acid, LTB4) and auto-oxidation products of docosahexaenoic acid (11-HDHA, 13-HDHA) were produced by aMDMs from patients with N-ERD compared with aMDMs from patients with NT CRSwNP (Fig 2, B and C; see Table E4, B, in this article’s Online Repository at www.jacionline.org). The synthesis of cysteinyl

---

**FIG 2.** Monocyte-derived macrophages from patients with N-ERD exhibit an increased capacity to produce proinflammatory mediators. A, Heat map of baseline chemokine production by N-ERD (n = 15), NT CRSwNP (n = 10) and healthy (n = 8) aMDMs, determined by multiplex cytokine array. B and C, Heat map (B) and bar graphs (C) of eicosanoids produced by aMDMs from patients with N-ERD (n = 13), those with NT CRSwNP (n = 10), or healthy individuals (n = 10), quantified by LC-MS/MS. Auto-scaled concentrations are shown as z score in blue (low) or red (high) or were not detected (ND). Data are shown as mean ± SD. *P < .05 (Kruskal-Wallis with Dunn multiple comparisons test). CysLTs, Cysteinyl LTs; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LXA4, lipoxin A4; oxoETE, oxo-eicosatetraenoic acid; TXB2, thromboxane B2.
FIG 3. N-ERD macrophages exhibit differential DNA methylation of genes involved in cell recruitment and acylcarnitine metabolism. A, Volcano plot of differentially methylated positions of N-ERD (n = 15) versus healthy (n = 8) aMDM; significant changes shown in blue (decrease) or red (increase); thresholds were set to $P_{\text{adj}} < 0.05$. B, Venn diagram of significant DEGs and DMRs in aMDMs and DMRs in monocytes (N-ERD vs healthy). C, Top 9 significant molecular function associations obtained by ToppGene analysis.
LTs, PGD₂, and thromboxane B₂ was increased, though not significantly, in N-ERD aMDMs. Thus, in addition to distinct proinflammatory gene expression profiles, macrophages from patients with N-ERD showed an elevated capacity to release PUFA metabolites and may thus contribute to proinflammatory eicosanoid profiles in N-ERD.

N-ERD macrophages display distinct DNA methylation and expression of chemokine and lipid metabolism genes

Because altered gene expression and eicosanoid production of N-ERD aMDMs persisted even after 7 days of in vitro differentiation, we next studied whether these changes were associated with epigenetic modifications.

Differentiation of CD14⁺ monocytes into aMDMs resulted in the increased methylation of genes involved in hematopoiesis (INPP5A, GPR171), while genes regulating macrophage recruitment and effector functions (HDMC5, IL7R, TGM2) were demethylated (see Table E6 in this article’s Online Repository at www.jacionline.org). Monocytes and aMDMs clustered according to cell type for both healthy and N-ERD, suggesting that differentiation of monocytes into aMDMs progressed normally for cells from patients with N-ERD (see Fig E2 in this article’s Online Repository at www.jacionline.org). However, when comparing aMDMs from patients with N-ERD and healthy individuals, we found 3930 lower and 211 higher differentially methylated positions (Fig 3, A; see Table E7, A, in this article’s Online Repository at www.jacionline.org). This was indicative of an overall transcriptionally preactivated macrophage phenotype in N-ERD.

Functional analysis of significant DMRs of both aMDMs and monocytes highlighted chemotaxis (CXCL2, PF4 [CXCL4]) and fatty acid/acylcarnitine metabolism (CPT1A, CPT1B, ACACA) as being associated with N-ERD (Fig 3, B and C, Table E7, B and C). While CPT1A, CPT1B, and ACACA were expressed at similar levels in aMDMs from patients with N-ERD and healthy individuals at baseline (Fig 3, D), CPT1A was significantly upregulated in N-ERD aMDMs as compared to healthy aMDMs, when stimulated with a mix of acylcarnitines (Fig 3, E). Together, this suggested that the persistent proinflammatory activation of N-ERD macrophages was at least partially due to metabolic and epigenetic changes related to fatty acid/acylcarnitine metabolism.

Acylcarnitines are increased in the upper and lower airways of patients with N-ERD

As our methylomics and RNAseq data suggested a role for metabolic changes in the proinflammatory reprogramming of N-ERD macrophages, we characterized lipid, amino acid, and carbohydrate metabolite profiles in nasal lining fluid and sputum of patients with N-ERD and those with NT CRSwNP or healthy controls by targeted metabolomics. Using a hierarchical clustering approach, 4 distinct metabolite clusters were identified in nasal lining fluid (Fig 4, A; see Table E8, A, in this article’s Online Repository at www.jacionline.org). The most prominent cluster 1 was associated with N-ERD and characterized by high concentrations of acylcarnitines, lysophosphatidylcholines, and histamines (Table E8, A). For sputum, hierarchical clustering resulted in 3 main metabolite clusters, with cluster 2 being associated with N-ERD and containing acylcarnitine metabolites (Fig 4, B, Table E8, B). Thus, N-ERD was characterized by elevated levels of acylcarnitines in the airways.

Plasma acylcarnitines and sphingolipids, but not adipokines are associated with N-ERD

To study whether local changes in metabolite profiles in N-ERD are reflected systemically, we additionally performed a metabolomics analysis of plasma from healthy individuals, patients with NT CRSwNP, and those with N-ERD. This analysis was extended to sphingolipids, which have previously been suggested as potential biomarkers of N-ERD. Hierarchical clustering differentiated the 3 patient groups into 5 partially overlapping clusters (Fig 4, C, Table E8, C). Clusters 1 and 2 mainly comprised amino acids with elevated levels in healthy controls, while clusters 3 and 4 were characterized by high concentrations of sphingolipids and sphingosine-1-phosphate in both patients with NT CRSwNP and those with N-ERD. Cluster 5, which was associated with N-ERD, contained sphingomyelins and medium- and long-chain acylcarnitines (C12–C16) (Fig 4, C, Table E8, C).

Further pathway analysis revealed the overall sphingolipid metabolism to be associated with N-ERD, although there were no significant differences for the individual metabolites (see Fig E3 and Table E9 in this article’s Online Repository at www.jacionline.org). Thus, N-ERD was characterized by both local and systemic aberrations in acylcarnitines and signaling lipids, suggesting a broad dysregulation of the lipid metabolism in this disease.

As acylcarnitines are increased in obesity-induced inflammation and an increased BMI had been suggested as a risk factor for N-ERD, we assessed the BMI and its potential correlation with clinical symptom scores. In addition, we quantified adipokines, which link obesity, asthma, and NT CRSwNP. However, patients with N-ERD showed no significant increase in BMI or leptin, and there was no correlation among BMI, adipokines, and SNOT-22 (Sino-Nasal Outcome Test) score (r = 0.25; P = .37) (see Fig E4, A–E, in this article’s Online Repository at www.jacionline.org). In contrast, adiponectin, which has been implicated in the proinflammatory activation of macrophages, tended to be increased in the plasma of patients with N-ERD compared with that of healthy controls and patients with NT CRSwNP (Fig E4, B).

Acylcarnitines are increased in N-ERD macrophages at baseline and on inflammatory challenge

As acylcarnitines had been implicated in the proinflammatory activation of macrophages, we studied whether intracellular acylcarnitine levels were altered in N-ERD macrophages. Indeed,
FIG 4. Multifluid metabolomics identifies alterations in the local, systemic, and intracellular acylcarnitine metabolism in patients with N-ERD. Multifluid metabolomics of nasal lining fluid (A), sputum (B), and plasma (C) from healthy individuals (n = 8, 3, 9, respectively), patients with NT CRSwNP (n = 8, 0, 10), or those with N-ERD (n = 11, 5, 12). Heat maps of metabolites assigned to clusters (see Table E8). Rows represent metabolites, and columns show normalized, autoscaled mean group concentrations. Concentration differences between patient groups are shown as Z score in blue (low) and red (high). D-E, Intracellular
most acylcarnitine species tended to be elevated in N-ERD aMDMs compared with healthy aMDMs (Fig 4, D, Table E8, D and E). In addition, some acylcarnitine species were further increased on LPS challenge, suggesting that aberrations in the macrophage acylcarnitine metabolism may be aggravated under inflammatory conditions (Fig 4, D and E, Table E8, D and E).

**N-ERD macrophages show increased inflammatory gene expression but similar bioenergetic reprogramming on LPS challenge**

To study potential proinflammatory effects of acylcarnitines, we stimulated aMDMs with myristoyl-L-carnitine (C14-carnitine), which had previously been reported to induce inflammatory gene expression in a murine macrophage cell line. However, in human aMDMs, C14-carnitine alone did not induce the expression of inflammatory cytokines (IL1B or TNFA), chemokines (CXCL2, CXCL8), or COX-2 (PTGS2) (Fig 5, A). In contrast, LPS alone or in combination with C14-carnitine showed a robust induction of these genes. Remarkably, N-ERD macrophages showed a significantly increased induction of CXCL2, IL1B, PTGS2, and TNFA on LPS stimulation, suggesting an increased responsiveness to inflammatory challenge (Fig 5, A).

As increased responsiveness to inflammatory stimuli (eg, LPS and β-glucan) has been linked to bioenergetic reprogramming, we further performed metabolic flux (Seahorse) assays to assess whether N-ERD macrophages exhibited altered bioenergetic profiles. At baseline, extracellular acidification rates as well as oxygen consumption rates of N-ERD aMDMs did not differ from those of healthy aMDMs, suggesting that there were no major changes in glycolysis or mitochondrial respiration (Fig 5, B–D). However, when stimulated with C14-carnitine plus LPS, aMDMs from healthy individuals but not from patients with N-ERD showed significant increases in mitochondrial respiration and glycolysis. Thus, rather than responding to an inflammatory stimulus with increased respiration and ATP production, N-ERD aMDMs readily increased the expression of proinflammatory effector molecules.

**N-ERD macrophages exhibit aberrant M2 activation**

As type 2 inflammation has classically been associated with M2 polarization of macrophages, we further analyzed M2 markers in N-ERD aMDMs at baseline and on inflammatory challenge. ALOX15, which is involved in the synthesis of proresolving mediators, tended to be reduced in N-ERD aMDMs while proinflammatory M2 markers (CCL17 and TGM2) tended to be increased (Fig 6, A). Stimulation with LPS resulted in a reduction of M2 marker genes (ALOX15, MRC1, TGM2), which was more prominent in aMDMs from patients with N-ERD (Fig 6, B). However, N-ERD aMDMs showed an exacerbated upregulation of the T2-promoting chemokine CCL17 in response to LPS + C14-carnitine (Fig 6, B). Thus, N-ERD aMDMs show a broad proinflammatory phenotype shift and may particularly exacerbate airway inflammation during an inflammatory or infectious challenge.

**DISCUSSION**

Chronic type 2 inflammatory airway diseases including N-ERD remain major unmet clinical needs. Macrophages are key players in type 2 inflammation, but the metabolic and epigenetic programs that determine macrophage phenotypes and functions in human patients remain largely unknown. The present study uncovers an unprecedented proinflammatory “macrophage memory” in patients suffering from N-ERD, a severe and therapy-resistant form of chronic airway inflammation. A combination of transcriptomics (RNAseq), methylomics, and multfluid targeted metabolomics (LC-MS/MS) analyses revealed distinct gene expression, DNA methyl- ation, and metabolite profiles in monocyte-derived macrophages from patients with N-ERD. In particular, N-ERD macrophages produced higher levels of proinflammatory fatty acid metabolites (acylcarnitines and 5-LOX products) and upregulated chemokines and cytokines more readily on inflammatory challenge. Strikingly, these increased proinflammatory capacities of N-ERD macrophages were apparent even after 7 days of *in vitro* differentiation. Our study thus identifies a persistent metabolic and epigenetic reprogramming of macrophages as an unprecedented pathomechanism of chronic type 2 inflammation.

We used aMDMs as an *in vitro* model because GM-CSF and TGFβ1 are essential for alveolar macrophage differentiation and as aMDMs share important characteristics with alveolar macrophages. Indeed, during inflammation, monocytes and MDM are highly relevant as monocytes infiltrate the airways and myeloid progenitors in the bone marrow can be functionally reprogrammed and respond more readily to inflammatory cues. The present study suggests that this phenomenon, referred to as “central trained immunity” may also contribute to chronic type 2 airway inflammation in N-ERD.

Recent studies revealed higher levels of acylcarnitines to be associated with increased fatty acid oxidation and airway inflammation in a murine model of allergic asthma. Here, we also found elevated levels of acylcarnitines in macrophages and body fluids of patients with N-ERD, suggesting an involvement of these metabolites in type 2 inflammation. However, changes in the acylcarnitine metabolism were not a sign of chronic airway inflammation per se as acylcarnitines were not altered in NT CRSwNP. Together with previously reported aberrations in the AA and sphingolipid metabolism, this suggests that the fatty acid metabolism is more globally dysregulated in N-ERD. Although altered metabolite profiles in body fluids from patients with N-ERD likely result from metabolic changes in multiple cell types, our findings support a role for macrophages in the production of acylcarnitines and proinflammatory 5-LOX metabolites during inflammation. Indeed, macrophages
**FIG 5.** N-ERD macrophages show increased responsiveness to LPS, but not acylcarnitine C14. A-D, Relative gene expression (A), oxygen consumption rates (OCRs) or extracellular acidification rates (ECARs) (B-D) of aMDMs (n = 6 healthy or n = 4-6 N-ERD) starved for 5 hours in complete medium (0.25% FBS) prior to stimulation with LPS (10 ng/mL), acylcarnitine C14 (10 μmol/L), or a combination of both for 19 hours. A, Relative gene expression of CXCL2, CXCL8, IL1B, PTGS2, and TNFA shown as fold change for healthy and N-ERD aMDMs (both n = 6). B and C, OCRs and ECARs measured in real time under basal conditions and in response to the indicated inhibitors. Representative graphs are shown for 1 healthy individual (n = 6 in group) and 1 patient with N-ERD (n = 4 in group). D, Basal respiration (Resp), basal ECAR, spare respiratory capacity, or ATP production of stimulated aMDMs. Data are shown as mean ± SD. *P < .05; **P < .01; ***P < .001 (repeated measures 2-way ANOVA with Sidak multiple comparisons test [A] or Wilcoxon test [B-D]). FCCP, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; max, maximum.
from patients with N-ERD exhibited a high capacity to generate proinflammatory 5-LOX products, including LTB4 and 5-oxo-eicosatetraenoic acid, which promote eosinophil activation and exacerbation in asthma. Furthermore, downregulation of CD1A–CD1C molecules (which promote host-protective molecules) has been described as an important mechanism of chronification and exacerbation. It remains to be studied whether this is a consequence of aberrant macrophage activation state. Indeed, a previous study suggested that patients with N-ERD are among the patients with the highest risk of multiple asthma exacerbations. However, in line with previous studies, sputum macrophages showed an abundant expression of proinflammatory chemokines, which represents an immune evasion mechanism that facilitates viral persistence. Thus, the gene expression profile of N-ERD aMDMs exhibited a distinct gene expression signature even after 7 days in vitro differentiation pointed toward an epigenetic reprogramming of these cells. Whole-genome methylation analysis revealed N-ERD-specific methylation signatures to be stable during monocyte-to-macrophage differentiation. However, differences in DNA methylation between N-ERD and healthy monocytes/macrophages were relatively small, suggesting that further epigenetic mechanisms (e.g., histone modifications) may contribute to the proinflammatory gene expression profiles identified by RNAseq. At baseline, N-ERD aMDMs only exhibited a moderate increase in their capacity to produce proinflammatory chemokines. Thus, the proinflammatory potential of N-ERD macrophages may only fully unfold in peripheral tissues, such as the nasal mucosa or lung, on inflammatory challenge. Unfortunately, low numbers of recovered sputum macrophages in healthy individuals precluded a comparison of airway macrophages from patients with N-ERD and healthy controls. However, in line with previous studies, sputum macrophages showed an abundant expression of proinflammatory chemokines (CXCL8–CXCL11), antigen-presenting molecules, immune regulatory enzymes (IDO-1, COX-2, microsomal PGE synthase I), AREG (AMPHIREGULIN), and SCGB1A1 as compared to aMDMs. Potentially as the result of active type 2 inflammation in the airways of patients with N-ERD, sMacs were also enriched in markers of M2 activation (IRF4, CCL17, IL4I1, ALOX15, HPGDS). Indeed, pathway analysis showed an enrichment for...
IL-4/IL-13 signaling and CCL17, an important driver of type 2 airway inflammation was more readily induced in N-ERD aMDMs.\textsuperscript{71,72} Importantly, overall disease burden, tissue composition, and metabolite profiles of patients with N-ERD and those with NT CRSwNP in our study were likely influenced by intranasal and/or inhaled corticosteroids and potentially by asthma, which was more frequent in N-ERD.\textsuperscript{1,73,74} Samples from untreated patients could potentially provide viable insights into macrophage activation during full-blown N-ERD, but such studies are difficult to realize due to the significant symptom burden of N-ERD. Based on the explorative design and strict safety rules in Germany, weaknesses of our study were the relatively small cohort size and the lack of confirmatory NSAID provocation. Thus, future studies should validate our findings in a larger N-ERD cohort, preferentially with a confirmed diagnosis of N-ERD after NSAID provocation. Such studies will hopefully shed further light on macrophage reprogramming as a pathomechanism of N-ERD. The inflammatory macrophage memory described here may also help to explain the acquired or delayed onset of the disease. Lastly, our work provides unprecedented insights into immune cell reprogramming during type 2 inflammation, which is likely relevant for multiple chronic airway diseases.

We thank Sandy Lösecke, Elisabeth Graf, and Thomas Schwarzmayr for RNAseq measurements performed at the Helmholtz Zentrum München, Institute of Human Genetics, Next-Generation Sequencing Core Facility; Jennifer Kriebel and Eva Reischl for genome-wide methylation measurements performed at the Helmholtz Zentrum München, Genome Analysis Center, Genotyping Core Facility; Julia Scarpa, Silke Becker, and Maria Kugler for metabolomics measurements performed at the Helmholtz Zentrum München, Genome Analysis Center, Metabolomics Core Facility; and Sandra Trautmann for metabolomics measurements performed at the Pharmacampus Frankfurt/Zentrum für Arzneimittelforschung, -Entwicklung und -Sicherheit (ZAPES), Institute of Clinical Pharmacology, Goethe University Frankfurt.

Clinical implications: The persistent proinflammatory activation of macrophages should be considered as a pathomechanism and therapeutic target in N-ERD.

REFERENCES


