

## ORIGINAL ARTICLE

Asthma and Lower Airway Disease



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# Sputum macrophage diversity and activation in asthma: Role of severity and inflammatory phenotype

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## Abstract

**Background:** Macrophages control innate and acquired immunity, but their role in severe asthma remains ill-defined. We investigated gene signatures of macrophage subtypes in the sputum of 104 asthmatics and 16 healthy volunteers from the U-BIOPRED cohort.

**Methods:** Forty-nine gene signatures (modules) for differentially stimulated macrophages, one to assess lung tissue-resident cells (TR-M $\phi$ ) and two for their polarization (classically and alternatively activated macrophages: M1 and M2, respectively) were studied using gene set variation analysis. We calculated enrichment scores (ES) across severity and previously identified asthma transcriptome-associated clusters (TACs).

**Results:** Macrophage numbers were significantly decreased in severe asthma compared to mild-moderate asthma and healthy volunteers. The ES for most modules were also significantly reduced in severe asthma except for 3 associated with inflammatory responses driven by TNF and Toll-like receptors *via* NF- $\kappa$ B, eicosanoid biosynthesis *via* the lipoygenase pathway and IL-2 biosynthesis (all  $P < .01$ ). Sputum macrophage number and the ES for most macrophage signatures were higher in the TAC3 group compared to TAC1 and TAC2 asthmatics. However, a high enrichment was found in TAC1 for 3 modules showing inflammatory pathways linked to Toll-like and TNF receptor activation and arachidonic acid metabolism ( $P < .001$ ) and in TAC2 for the inflammasome and interferon signalling pathways ( $P < .001$ ). Data were validated in the ADEPT cohort. Module analysis provides additional information compared to conventional M1 and M2 classification. TR-M $\phi$  were enriched in TAC3 and associated with mitochondrial function.

**Conclusions:** Macrophage activation is attenuated in severe granulocytic asthma highlighting defective innate immunity except for specific subsets characterized by distinct inflammatory pathways.

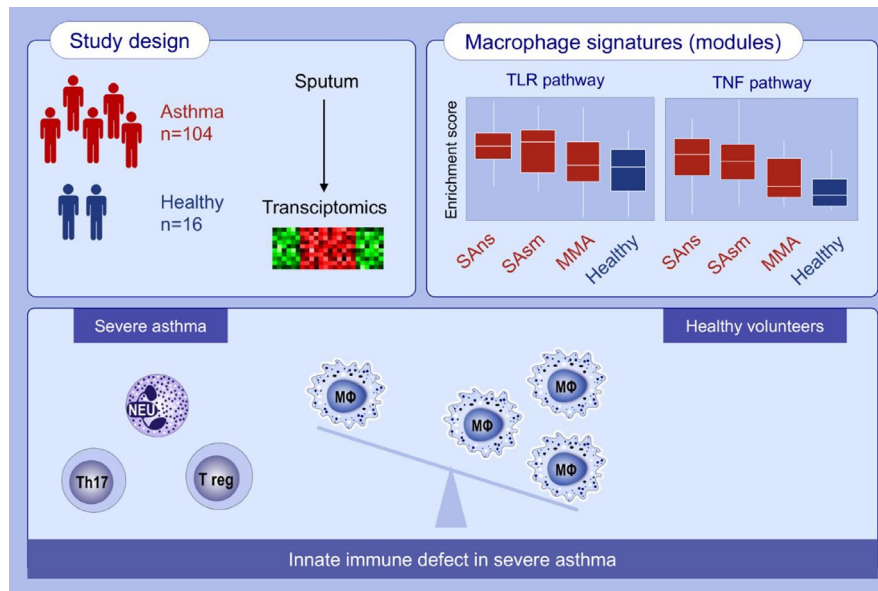
Consortium project team members are listed under Acknowledgments

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## KEYWORDS

asthma, gene set variation analysis, macrophage subtypes, sputum, tissue-resident



## GRAPHICAL ABSTRACT

This study investigates gene signature of macrophage phenotypes in sputum of asthmatic and healthy controls. Several specific macrophage subsets identified by gene signatures are highly activated in severe granulocytic asthma and involved distinct inflammatory pathways (eg tumour necrosis factor [TNF] and Toll-like receptor [TLR] signalling pathways). Macrophage activation and numbers are reduced in severe granulocytic asthma highlighting defective innate immunity.

Abbreviations: MMA, mild/moderate asthmatics; SAns, severe asthmatics nonsmokers; SAsm, severe asthmatics smokers; TLR, Toll-like receptor; TNF, tumor necrosis factor

## 1 | INTRODUCTION

Asthma is a heterogeneous disease driven by diverse inflammatory mechanisms, which presents in various clinical forms. Severe asthma is a clinically defined subset of asthma that remains partly or totally unresponsive to known asthma treatments.<sup>1</sup> Both recruited inflammatory and resident airway structural cells participate in the inflammatory and remodelling processes associated with asthma phenotypes.<sup>2</sup> Recently, type-2-related and non-type-2-related molecular phenotypes or transcriptomic-associated clusters (TACs) have been described.<sup>3</sup> TACs were derived from clustering of differentially expressed genes identified in the sputum of asthmatic patients and provide mechanistic pathways beyond eosinophilic subtyping.

Macrophages within the lung play a role in the control of both innate and acquired immunity, tissue homeostasis, angiogenesis and metabolism.<sup>4</sup> Hallmarks of macrophages are their plasticity and diversity. Two populations of macrophages coexist in the lung: (a) tissue-resident macrophages (TR-Mφ) that arise during embryogenesis are associated with proliferation and defined by Krebs cycle, amino acid and fatty acid (FA) metabolic processes and (b) recruited macrophages that originate postnatally from circulating monocytes and are involved in immune signalling and inflammation and defined by glycolysis and arginine metabolism.<sup>5</sup> Tissue damage or infection results in TR-Mφ activation with production of inflammatory mediators, together with

monocyte recruitment.<sup>4,6</sup> Cytokines drive macrophage polarization (diversity) with two classical subtypes according to their functional properties: classically activated M1 driven by IFN- $\gamma$ , LPS, GM-CSF or TNF- $\alpha$  exposure and alternatively activated M2 macrophages induced by IL-4, IL-10, IL-13, glucocorticoids or TLR ligands.<sup>4</sup> While M1 macrophages have pro-inflammatory properties and are associated with Th1 responses, M2 macrophages control inflammation through the high expression of IL-10 and are involved in Th2-mediated immunity.<sup>4,7</sup>

The role of macrophages in asthma pathogenesis is unclear because of the limited methods available to characterize them.<sup>6</sup> Current data suggest that the presence of M1 macrophages is associated with disease progression and airway remodelling, M2 macrophages are associated with type-2 asthma<sup>4,8</sup> and the role of TR-Mφ remains unknown. However, human monocyte-derived macrophages stimulated with 28 different stimuli produced 49 distinct transcriptomic modules highlighting the spectrum of macrophage diversity.<sup>9</sup>

We hypothesized that analysis of these 49 macrophage transcriptomic signatures will indicate activation of distinct macrophage subsets in severe asthma. We assessed this using gene set variation analysis (GSVA) using sputum cells from the U-BIOPRED (Unbiased BIOMarkers in PREDiction of respiratory disease outcomes) asthma cohort<sup>10</sup> with validation in the ADEPT (Airway Disease Endotyping for Personalized Therapeutics) cohort.<sup>11</sup> This is the first comprehensive analysis of airway macrophage subtypes in asthma.

**TABLE 1** Characteristics of study participants from U-BIOPRED cohort providing sputum transcriptomics

	Severe asthma		Mild-moderate asthma	Healthy volunteer	P-value
	Nonsmoking	Smoking			
Subjects n	61	23	20	16	
Age years	53.89 ± 12.14	52.65 ± 11.15	42.05 ± 15.85	38.06 ± 13.27	<.001
Female n (%)	37 (60.66)	12 (52.17)	11 (55)	4 (25)	<.001
BMI kg/m <sup>2</sup>	27.90 ± 5.26	29.40 ± 4.98	25.76 ± 4.96	25.71 ± 2.79	.034
Current smoking n (%)	0 (0)	8 (34.78)	0 (0)	0 (0)	<.001
Atopy n (%)	42 (68.85)	13 (56.52)	16 (80)	5 (31.25)	<.001
Comorbidities					
Allergic rhinitis n (%)	24 (39.34)	7 (30.43)	11 (55)	2 (12.50)	.020
Eczema n (%)	17 (27.87)	9 (39.13)	7 (35)	0 (0)	.046
Nasal polyposis n (%)	22 (36.07)	8 (34.78)	4 (20)	1 (6.25)	.049
Oral corticosteroids daily	22 (36.07)	12 (52.17)	0 (0)	NA	<.001
Exacerbations number/year	2.41 ± 2.01	2.35 ± 2.59	0.45 ± 0.76	NA	<.001
ACQ5	2.26 ± 1.31	2.14 ± 1.20	1.00 ± 0.75	NA	<.001
Lung function					
FEV1% predicted	61.25 ± 21.66	67.87 ± 18.01	92.45 ± 13.00	105.90 ± 10.29	<.001
FVC % predicted	87.25 ± 18.53	95.38 ± 19.37	108.57 ± 14.32	113.58 ± 10.49	<.001
FEV1/FVC ratio	0.55 ± 0.13	0.59 ± 0.09	0.71 ± 0.08	0.79 ± 0.03	<.001
FeNO ppb	40.84 ± 39.64	27.04 ± 19.67	40.37 ± 33.92	19.71 ± 9.86	<.001
Sputum cells (%)					
Eosinophils	13.93 ± 21.12	12.83 ± 15.72	1.82 ± 3.07	0.12 ± 0.25	<.001
Neutrophils	60.09 ± 27.73	55.97 ± 19.65	50.01 ± 25.48	43.16 ± 25.85	.030
Lymphocytes	1.26 ± 1.34	1.39 ± 0.88	1.11 ± 1.17	1.26 ± 1.01	.987
Macrophages	24.69 ± 21.47	29.78 ± 19.49	47.02 ± 24.45	55.45 ± 25.67	<.001
Mast cells	0.03 ± 0.08	0.02 ± 0.08	0.04 ± 0.08	0.01 ± 0.05	.404
Sputum cells count ×10 <sup>3</sup> /μL					
Total cells	590.51 ± 122.94	602.29 ± 93.99	614.50 ± 91.01	680.47 ± 149.15	.004
Eosinophils	71.03 ± 107.72	69.70 ± 86.61	10.45 ± 17.89	0.63 ± 1.31	<.001
Neutrophils	315.90 ± 149.14	297.91 ± 104.02	270.55 ± 252.50	228.81 ± 140.25	.039
Macrophages	132.36 ± 123.50	158.26 ± 103.48	253.45 ± 133.56	289.13 ± 133.67	<.001
Blood cells ×10 <sup>3</sup> /μL					
Eosinophils	0.38 ± 0.33	0.30 ± 0.15	0.21 ± 0.11	0.13 ± 0.09	<.001
Neutrophils	5.20 ± 2.35	5.99 ± 2.42	3.69 ± 1.22	3.85 ± 1.55	.009
Serum markers					
Total IgE IU/mL	234.14 ± 417.61	377.14 ± 513.62	336.04 ± 861.59	113.66 ± 172.99	.089
Periostin ng/mL	53.01 ± 18.45	51.49 ± 17.94	45.79 ± 10.77	47.33 ± 5.38	.059
IL-13 pg/mL	1.07 ± 1.56	0.63 ± 0.38	0.68 ± 0.44	0.36 ± 0.21	.003
Eotaxin pg/mL	140.84 ± 80.77	109.35 ± 41.20	92.06 ± 45.18	92.19 ± 37.03	.001
TNF-α pg/mL	1.97 ± 0.57	1.96 ± 0.65	1.88 ± 0.79	1.71 ± 0.33	.028
IL-6 pg/mL	1.33 ± 1.22	1.05 ± 0.49	0.83 ± 0.73	0.52 ± 0.26	<.001
IFN-γ pg/mL	9.99 ± 9	7.84 ± 7.49	9.81 ± 7.50	6.95 ± 3.86	0.057
MCP1 pg/mL	122.18 ± 38.02	113.25 ± 42.32	92.02 ± 31.14	85.16 ± 20.19	<.001
CCL18 pg/mL	217.04 ± 125.40	193.78 ± 70.60	148.41 ± 109.45	107.20 ± 58.76	<.001
CRP mg/L	8.31 ± 16.18	3.17 ± 3.01	2.04 ± 2.74	0.89 ± 0.73	<.001

Note: Data are presented in mean ± SD.

Abbreviations: ACQ5, Asthma Control Questionnaire 5; BMI, body mass index; CCL18, C-C motif chemokine ligand 18; CRP, C-reactive protein; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1s; FVC, forced vital capacity; IFN-γ, interferon gamma; Ig E, immunoglobulin E; IL-13, interleukin 13; IL-6, interleukin 6; MCP1, monocyte chemoattractant protein 1; NA, not applicable; TNF-α, tumour necrosis factor.

**TABLE 2** Description of macrophage modules according to stimuli and corresponding pathways and functions (KEGG 2019, Reactome 2016, WikiPathways Humans 2019)

Module	Stimuli	Function
1	Fatty acids (LA, OA)	Glycolysis/gluconeogenesis, metabolism of carbohydrates, biosynthesis of amino acids
2	Fatty acids (LA)	Methionine de novo and salvage pathway, cysteine and methionine metabolism, ribosome biogenesis
3	M1 (LPS, LPS + IFN- $\gamma$ )	Vesicle biogenesis by lysosome and vesicle-mediated transport, development of macrophages and dendritic cells, IL-6 signalling pathway
4	Fatty acids (OA, PA)	Mitochondrial fatty acid beta-oxidation of saturated fatty acids, ligand-independent caspase activation, TRAF6-mediated activation in TLR7/8 or 9, regulation of neutrophil-mediated immunity and degranulation
5	Fatty acids (PA, OA, LA)	Protein processing in endoplasmic reticulum, base excision repair, cellular response to sterol depletion
6	Fatty acids (OA, LA, PA)	Carbohydrate metabolism, negative regulation of leucocyte leucocytes degranulation, positive regulation of IL-8 secretion
7	M1 (IFN- $\gamma$ )	Interferon-signalling pathways, antiviral mechanism by interferon stimulated genes, IL-10 anti-inflammatory signalling pathway
8	M1 (IFN- $\gamma$ , IFN- $\gamma$ + TNF- $\alpha$ )	Phagosome, type II interferon signalling pathway (IFN- $\gamma$ ), Toll-like receptor signalling pathway
9	M1 (IFN- $\gamma$ + TNF- $\alpha$ , IFN- $\gamma$ )	Aminoacyl-tRNA biosynthesis, signalling by WNT, positive regulation of TNF-mediated signalling pathway
10	M2 (IL-4, IL-10)	Ribosome, electron transport chain in mitochondria, interferon type I signalling pathway
11	M2 (IL-4, IL-13)	Modulation of viral transcription, ribosome
12	M2 (IL-4)	Galactose, fructose, mannose metabolism, target of rapamycin (mTOR) signalling pathway, regulation of TP53 activity
13	M2 (IL-4, IL-13)	Oxidative phosphorylation and citrate cycle in mitochondria, regulation of leucocyte activation, Toll-like receptor 2 signalling pathway, positive regulation of TNF- $\alpha$ biosynthesis
14 (SAsm)	M2 (IL-4, IL-13)	<b>Phagosome-lysosome fusion (<math>r = .60, P &lt; .001</math>)</b>
15	M2 (IL-4, IL-13)	<b>Inflammatory response, neutrophil chemotaxis (<math>r = .55, P &lt; .001</math>)</b> <b>Toll-like receptors (1, 2, 4, 6) pathway (<math>r = .67, P &lt; .001</math>)</b> <b>IL-17 signalling pathway (<math>r = .34, P &lt; .001</math>)</b> <b>Arachidonic acid metabolism (<math>r = .28, P = .002</math>)</b>
16	Fatty acids (PA, SA)	Cholesterol metabolism, PPAR signalling pathway, IL-1 $\beta$ induced activation of NF- $\kappa$ B pathway
17	Fatty acids (PA, SA)	Glycosphingolipid biosynthesis
18	Fatty acids (PA, LiA, OA)	Proteasome, regulation of apoptosis
19	Fatty acids (PA, SA, OA)	Proteasome, phagosome
20	Fatty acids (PA, SA, LiA)	Positive regulation of cholesterol storage, positive regulation of T-helper cell differentiation, IL-10 anti-inflammatory signalling pathway
21	Fatty acids (PA, SA)	<b>Eicosanoid biosynthetic process on the lipoxygenase pathway, Unsaturated fatty acids biosynthesis (<math>r = .53, P &lt; .001</math>)</b>
22	Fatty acids (OA, LA, LiA)	Cell cycle, mitosis
23	Fatty acids (OA, LA, LiA)	Methylation pathway, mitochondrial complex I assembly model OXPHOS system, IL-1 $\beta$ induced activation of NF- $\kappa$ B pathway
24	Fatty acids (OA, LA, LiA)	rRNA metabolic process, PI3K-AKT-mTOR signalling pathway, Mitochondrial translation
25	Fatty acids (PA, LiA, SA)	Golgi vesicle transport, protein transport
26	Fatty acids (LiA, OA, PA)	RNA binding, NF- $\kappa$ B binding
27	Fatty acids (LA, LA, SA)	Regulation of steroid and cholesterol biosynthesis
28	Fatty acids (OA, LiA)	Phagosome-lysosome fusion, regulation of Toll-like receptor 9 signalling pathway, positive regulation of granulocyte differentiation
29	Others (TPP, TPP + IFN)	<b>NF-<math>\kappa</math>B signalling pathway (<math>r = .62, P &lt; .001</math>),</b> <b>TNF-<math>\alpha</math> signalling pathway (<math>r = .50, P &lt; .001</math>),</b> <b>Positive regulation of IL-2 biosynthetic process (<math>r = .66, P &lt; .001</math>)</b>
30	Others (TPP, TPP + IFN)	Positive regulation of acute inflammatory response, regulation of chemokines production, cytokine-cytokine receptor interaction and neutrophil-mediated immunity

(Continues)

TABLE 2 (Continued)

Module	Stimuli	Function
31	Fatty acids (PA, SA)	Toll-like receptor signalling pathway, TNF- $\alpha$ signalling pathway, IL-1 $\beta$ signalling pathway
32	Others (TPP, TPP + IFN)	Fatty acids, triacylglycerol and ketone metabolism, regulation of neutrophil-mediated immunity and degranulation, regulation of IL-1 $\beta$ production and NK cell mediated cytotoxicity
33	M1 (LPS)	IL-2 signalling pathway, T-cell antigen receptor (TCR) pathway, intrinsic apoptosis in response to oxidative stress
34	Others (TPP, TPP + IFN)	Oxidative phosphorylation (OXPHOS) in mitochondria, modulation of TGF $\beta$ signalling pathway, positive regulation of adaptive immune response
35	M1 (IFN- $\gamma$ + TNF- $\alpha$ )	OXPHOS system in mitochondria (electron transport)
36	Others (TPP)	Staphylococcus aureus infection, phagosome
37	M2 (IL-4)	Proteasome degradation, inositol phosphate metabolic process
38	Others (TPP, TPP + IFN)	Positive regulation of microtubule activity, snRNA metabolic process
39	Others (TPP, TPP + IFN)	Glycolysis/gluconeogenesis, amino acid metabolism, steroid catabolic process
40	Others (TPP, TPP + IFN)	Ribosome biogenesis, DNA replication
41	Others (GC)	Sphingolipid metabolic pathway, apoptotic cell clearance, insulin-like growth factor (IGF1)-Akt signalling pathway
42	Others (GC)	Lysosome, Toll-like receptor pathway, carbohydrate digestion and absorption
43	Others (GC, HDL)	Complement and coagulation cascades, superoxide metabolic process, regulation of lymphocyte activation
44	Fatty acids (OA)	TGF $\beta$ -signalling pathway, regulation of IL-10 secretion, mitochondrial DNA metabolic process
45	M2 (IL-4, IL-10, IL-13)	NF- $\kappa$ B signalling pathway, TNF signalling pathway, fatty acids biosynthesis
46	M2 (IL-4, PGE2)	Antigen processing and presentation via MHC class II, Th-17 differentiation, allograft rejection
47	M2 (IL-4, PGE2)	DNA damage induced protein phosphorylation, positive regulation of extracellular matrix assembly, TP53 network
48	M2 (IL-4, IL-13, PGE2)	Ions homeostasis, Toll-like receptor signalling pathway, angiogenesis
49	Unstimulated	Negative regulation of acute inflammatory response, negative regulation by host of viral process, intrinsic apoptotic signalling pathway in response to oxidative stress

Note: Modules enriched in severe asthma are highlighted in BOLD with *r* and *P* values indicated.

Abbreviations: IFN, interferon; IL-13, interleukin 13; IL-17, interleukin 17; IL-2, interleukin 2; IL-4, interleukin 4; M2, alternatively polarized macrophage; NF- $\kappa$ B, nuclear-factor kappa B; PA, palmitic acid; SA, stearic acid; SAsm, severe asthma smokers and ex-smokers; TNF, tumour necrosis factor alpha; TPP, tumour necrosis factor alpha + prostaglandin E2 + Toll-like receptor 2 ligand.

## 2 | METHODS

### 2.1 | Study population and design

We studied data from participants of the U-BIOPRED adult cohort<sup>10</sup> who underwent sputum cell transcriptomic analysis.<sup>3</sup> This included 104 asthmatics: mild-moderate asthma ( $n = 20$ ), nonsmoking severe asthmatics ( $n = 61$ ) and current or ex-smokers with severe asthma ( $n = 23$ ), and 16 healthy volunteers (Table 1). All participants gave written informed consent. For the asthmatic patients, the analyses were done also according to the presence of airflow limitation defined as FEV1/FVC < 0.70 and FEV1 < 75% of predicted.<sup>3</sup>

A description of the analytical plan is provided in Figure S1.

### 2.2 | Microarray analysis of sputum transcriptomes

Sputum induction<sup>12</sup> and transcriptomic analysis has been described previously.<sup>13</sup> In brief, sputum cells were obtained from

plugs obtained following inhalation of hypertonic saline. Gene expression profiling was performed on extracted RNA using microarrays.<sup>3</sup> A comparison of sputum gene expression profiles from asthmatics with high ( $\geq 1.5\%$ ) or low ( $< 1.5\%$ ) sputum eosinophilia and the healthy volunteer group identified 508 differentially expressed genes. Batch/technical effects, age, sex and administration of oral corticosteroid were adjusted for as covariates. Unsupervised hierarchical clustering based on Euclidean distance on these 508 genes identified 3 transcriptomic-associated clusters (TACs): TAC1 was associated with Th2 inflammation, sputum eosinophilia, more severe asthma, high rate oral corticosteroid dependency, frequent exacerbations and severe airflow obstruction, TAC2 with IFN- $\gamma$ , TNF- $\alpha$  and inflammasome-signalling pathways, sputum neutrophilia, high prevalence of eczema and serum C-reactive protein levels and TAC3 with paucigranulocytic inflammation, metabolic pathways and better preserved lung function.<sup>3</sup> These groups are considered as reflective of different molecular phenotypes of asthma in the U-BIOPRED cohort and were used in our analyses.

### 2.3 | Gene set variation analysis (GSVA)

Gene set variation analysis is a gene set enrichment method that estimates the variation in a pathway activity across a sample population in an unsupervised manner. It is used to detect changes in pathway or gene signature activity over a sample population and indicates differences within populations and between groups of subjects.<sup>13,14</sup> GSVA calculates sample-wise enrichment scores (ES) across the whole data set with a range from -1 to +1.

### 2.4 | Macrophage signatures

Forty-nine distinct modules previously published<sup>9</sup> derived from 28 distinct treatments (eg IFN- $\gamma$ , IL-4, IL-10, LPS, TNF- $\alpha$ , IL-13, free fatty acids, high-density lipoprotein, prostaglandin E2, corticosteroids or various association of these stimuli), containing 27 to 884 genes per module, were manually curated in R Bioconductor (R Foundation for Statistical Computing, Vienna, Austria) to analyse macrophage signatures according to asthma severity, TACs and airflow limitation. Validated M1 and M2 signatures (stimulated by IFN- $\gamma$  + LPS or TNF- $\alpha$ , and IL-4 + IL-13, respectively)<sup>15</sup> and a TR-M $\phi$  signature adapted from a murine model<sup>16</sup> were also examined (Table S1). Although some of the stimuli were the same, the module genes were often different reflecting altered cellular states resulting from distinct pathways characterizing the modules and highlighting macrophage heterogeneity (Table 2). Validation of the module signatures was performed using GSE90010 comparing purified MDMs populations against alveolar macrophages (AM) from idiopathic pulmonary fibrosis (IPF) patients (Table S2).

### 2.5 | Pathway analysis

The functions and pathways for each gene signature were assessed using KEGG 2019, WikiPathways 2019 and Reactome 2016 databases within Enrichr<sup>17</sup> (Table 2), and for the pooled activated genes by STRING (www.string-db.org).

### 2.6 | Statistical analysis

A linear model for microarray data (Bioconductor R package limma) with a false discovery rate (FDR) correction was used for differential gene expression analysis and recursive partitioning (party package in R) for decision tree learning. An FDR  $\leq 0.05$  was considered statistically significant. The ES was calculated for each gene set for each subject. ANOVA was used to analyse ES differences between group means, and the Student *t* test was applied to compare ES differences between two means.

## 3 | RESULTS

### 3.1 | Macrophage signatures according asthma severity

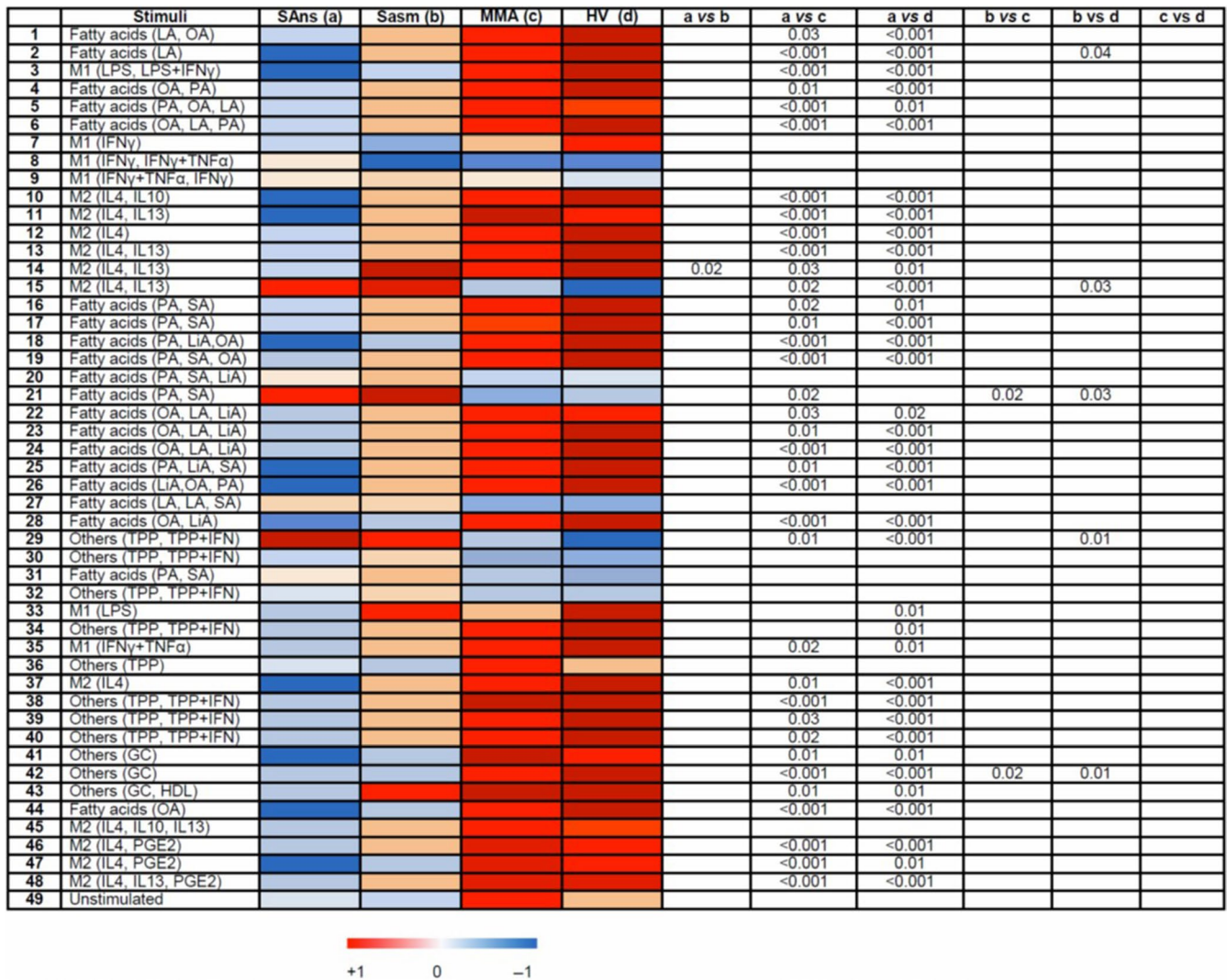
In initial analysis to determine whether macrophage modules were selective for macrophages, we demonstrated that 41/49 module signatures were similarly enriched in AM and MDMs from healthy volunteers and IPF patients (Table S2). Two of the signatures were significantly decreased while 6 of the signatures were significantly enriched in AM compared with monocyte-derived macrophages.

The total number and percentage of macrophages in sputum was reduced by ~50% in severe asthma but not in mild-moderate asthma (Table 1). However, not all macrophages subtypes were decreased according to GSVA analysis. Figure 1 represents the ES values for the 49 macrophage modules showing a significant reduction in the ES for most macrophage modules in severe asthma vs healthy volunteers. The ES for modules 7, 8, 9, 20, 27, 30-32, 36 and 45 which are associated with antiviral activity by IFN-stimulated genes, phagosome activation and FA metabolism were similar between asthma and healthy volunteers (Figure 1). The most significant biological pathways and functions ascribed to each module are summarized in Table 2 with the respective *r* and *P* values for modules 15, 21 and 29, which were significantly enriched in severe asthma, also being shown.

Representative dot plots of the GSVA ES for selected modules are shown in Figure 2 to indicate the range of enrichment within subject groups. In contrast to the reduced numbers of macrophages seen in severe asthma compared with mild-moderate asthma and healthy volunteers, modules 15, 21, respectively 29 were significantly enriched in severe asthma vs mild-moderate asthma and healthy volunteers (Figures 1 and 2A,B). These modules reflect IL-4/IL-13 activation (module 15), FA (stearic and palmitic acid)-stimulated macrophages (module 21) and macrophages associated with chronic inflammation (CI-M $\phi$ ) (module 29, stimulated by TPP: TNF- $\alpha$  + PGE2 + the TLR2-ligand Pam3CSK4).<sup>9</sup> Their genes are involved in innate inflammatory responses, eicosanoid biosynthesis via the 5-LOX pathway, T-cell differentiation through the regulation of IL-2 production and IL-17 signalling (Table 2). Only module 14, associated with the phago-lysosome fusion, lymphocyte migration and fat cell proliferation, was significantly enriched in severe asthma smokers vs severe asthma nonsmokers (*P* = .02). In contrast, representative GSVA plots of the enrichment of module 18 (FA) show significant down-regulation in severe asthma compared to mild-moderate asthma and healthy volunteers (Figure 2C). There were no significant differences in the enrichment of TR-M $\phi$ , M1 or M2<sup>14,15</sup> or in sputum across asthma severity using GSVA (Figure 2D-F).

These data highlight the plasticity of macrophages in that macrophage subtypes or states distinguished by WGCNA modules provide additional signals compared to validated M1 and M2 signatures. Overall, the data indicate reduced macrophage numbers





**FIGURE 1** Heat map of enrichment scores (ES) for the 49 macrophage activation modules according to clinical category. GC, glucocorticoids; HDL, high-density lipoprotein; HV, healthy nonsmoking controls; IFN- $\gamma$ , interferon  $\gamma$ ; IL-10, interleukin 10; IL-13, interleukin 13; IL4, interleukin 4; LA, lauric acid; LiA, linoleic acid; LPS, bacterial lipopolysaccharide; MMA, mild/moderate asthma; OA, oleic acid; PA, palmitic acid; PGE2, prostaglandin E2; SA, stearic acid; SAns, severe asthma nonsmokers; Sasm, severe asthma smokers; TNF- $\alpha$ , tumour necrosis factor; TPP, TNF- $\alpha$  + prostaglandin E2 + Toll-like receptor 2 ligand. Where no value is given the results were not significant

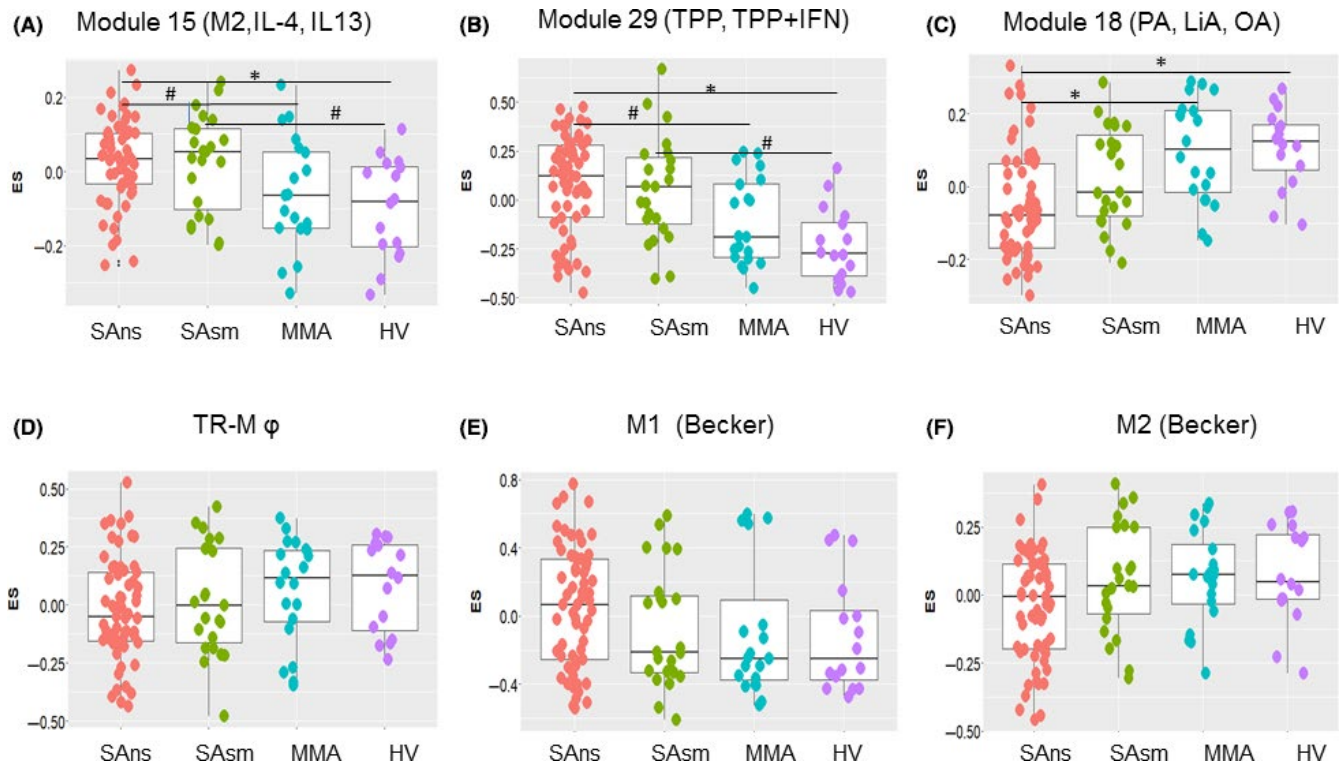
and activation status in severe asthma compared to mild-moderate asthma and healthy volunteers suggesting an attenuated innate immune response in these patients. However, there is a limited sub-type-specific activation of macrophages in severe asthma compared to mild-moderate asthma.

### 3.2 | Macrophage enrichment according to sputum granulocytes

When the sputum transcriptome was assessed according to TAC signature as representative of sputum asthma molecular phenotypes, there was a significant decrease of the macrophage absolute cell count in TAC1 ( $141.53 \times 10^3/\mu\text{L}$ ) compared to TAC2 ( $195.46 \times 10^3/\mu\text{L}$ ) and TAC3 ( $202.88 \times 10^3/\mu\text{L}$ ) subjects ( $P = .009$  and  $P = .003$ , respectively) (Table S3) without significant difference between TAC2

and TAC3 ( $P = .705$ ). The ES of most (35/49) macrophage modules were increased in TAC3 compared to TAC1 and TAC2 (Figure 3) while only 4 modules being significantly enriched in TAC1 compared to TAC3. These were module 15 implicated in positive regulation of inflammatory response by neutrophil chemotaxis, arachidonic acid metabolism, activation of TLR and IL-17 signalling pathways; module 21 associated with eicosanoids (via 5-LOX pathway) and unsaturated FA biosynthesis; module 27 including genes that regulate steroid and cholesterol biosynthesis and module 29 characterized by genes encoding TNF- $\alpha$  and NF- $\kappa\text{B}$  pathways and positive regulation of IL-2 production.

In TAC2, the modules 15, 27 and 29 are also highly enriched compared to TAC3 as well the modules 7-8 associated with antiviral activity via the IFN- $\gamma$  pathway; module 20 with IL-10 signalling pathway and Th-cell differentiation; modules 30-32 with the inflammatory response with neutrophil chemotaxis involving



**FIGURE 2** Representative analysis of selected macrophage module enrichment scores (ES) according asthma severity. Gene set variation analysis (GSVA) plots of individual subject ES for (A) module 15—IL-4- and IL-13-stimulated, (B) module 29—TPP- and TPP + IFN-stimulated and (C) module 18—PA-, LiA- and OA-stimulated. The ES for tissue-resident macrophages (D, TR-M $\phi$ ), classically activated macrophages (E, M1 from Becker) and alternatively activated macrophages (F, M2 from Becker) are also shown. HV, healthy volunteers; IFN- $\gamma$ , interferon  $\gamma$ ; IL-13, interleukin 13; IL-4, interleukin 4; LiA, linoleic acid; MMA, mild/moderate asthma; OA, oleic acid; PA, palmitic acid; SAns, severe asthma nonsmokers; SAsm, severe asthma smokers and ex-smokers; TPP, TNF- $\alpha$  + prostaglandin E2 + Toll-like receptor 2 ligand (Pam3CSK4). \* $P < .001$ ; # $P < .05$ ;  $P = NS$  (NS, nonsignificant)

TLR, inflammasome and TNF- $\alpha$  signalling pathways; and modules 47 and 49 with mitochondrial process and apoptosis secondary to the oxidative stress.

We observed an enrichment of module 15 in both TAC1 (eosinophilic) and TAC2 (neutrophilic) groups. However, other M2-related signatures such as module 47 (IL-4, PGE<sub>2</sub>) was enriched in TAC2 compared to TAC1 and module 48 (IL-4, IL-13 and PGE<sub>2</sub>) was enriched in TAC3. This suggests that these M2-related subtypes reflect the local inflammatory milieu and that not all M2 cells are the same. In contrast, TAC2 was more enriched with macrophage signatures encoding M1-like modules (modules 7 and 8) and macrophages stimulated by either FAs (modules 20, 21, 27 and 31) or TPP (modules 29, 30 and 32) (Figure 3). This indicates either the presence of low-grade sub-clinical infection or of a skewed immune response following previous infection since no overt infection had been reported in the 3 months prior to sputum induction.

Representative dot plots of the GSVA ES for selected modules indicate the range of enrichment within subject groups. Module 21 (FA) was significantly down-regulated in TAC2 compared with TAC1 and TAC3 (Figure 4A), module 29 (TPP) was significantly enriched in TAC1 and TAC2 compared with TAC3 (Figure 4B) while module 30 (a different TPP module) (Figure 4C) was enriched in TAC2 compared to both TAC1 and TAC3.

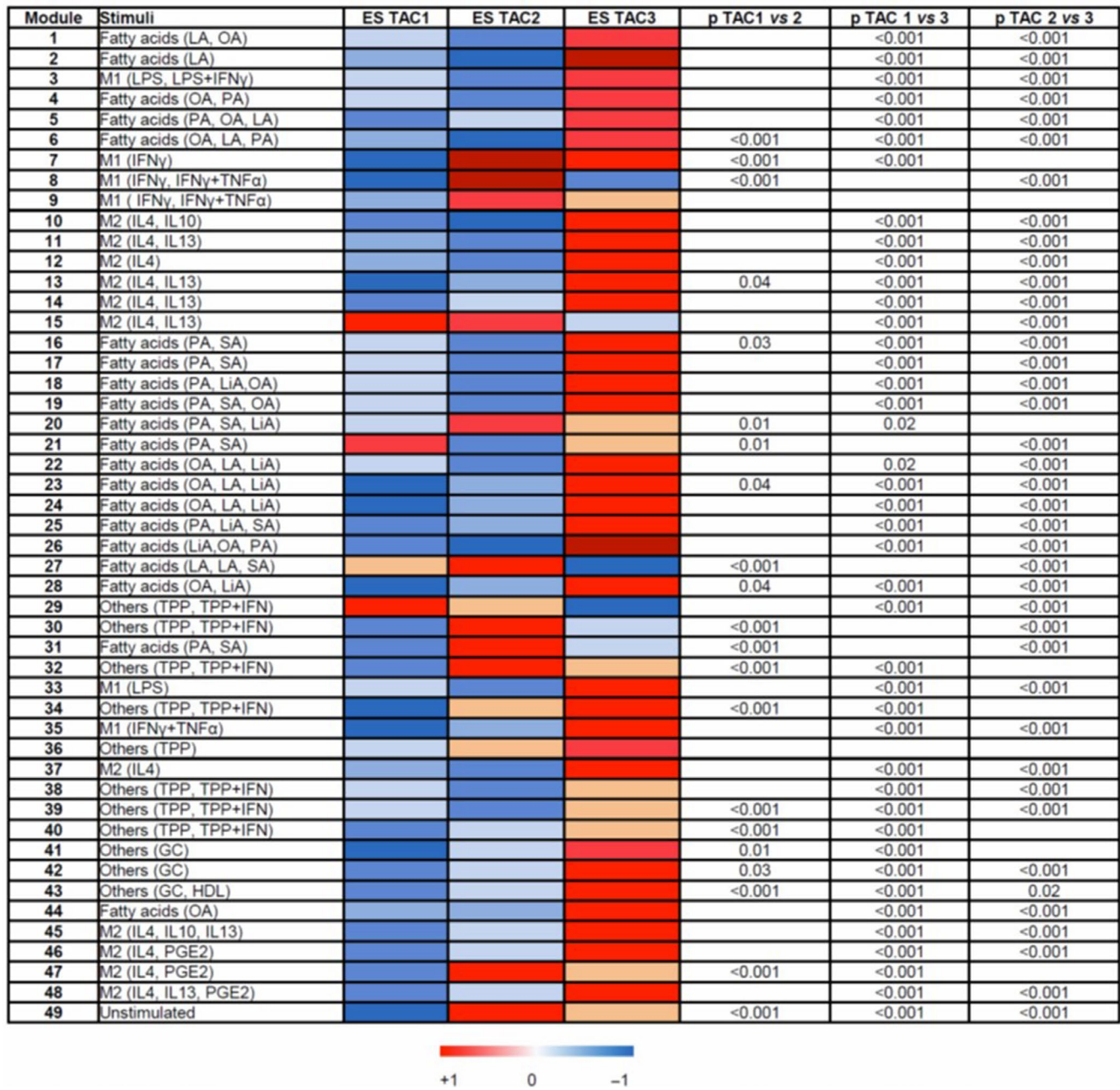
In contrast, the TR-M $\phi$  macrophage signature was significantly enriched in the TAC3 module (Figure 4D), the M1 validated

signature<sup>14</sup> in TAC2 (Figure 4E) and the M2 validated signature<sup>14</sup> was significantly reduced in TAC2 (Figure 4F). The genes from TR-M $\phi$  signature<sup>15</sup> encode proteins involved in metabolic process, tissue homeostasis and immunity (Table S4).<sup>16,18</sup>

The M1 validated signature was highly correlated with sputum neutrophilia (TAC2), with T naïve cells and with pathways involved in inflammation (NF- $\kappa$ B, TNF, TLR and inflammasome pathways) (Table S5). The M2-validated signature was significantly correlated with TAC3 and the TR-M $\phi$  signature with TAC3, to phago-lysosome activity and to oxidative phosphorylation (OXPHOS) (Table S5). More detailed information about TR-M $\phi$  functions is provided in the (Table S6).

Overall, these data suggest that patients with severe granulocytic asthma have a reduced innate immune response as indicated by the reduced enrichment of most macrophage subsets. However, a select number of macrophage modules/subsets are enhanced in TAC1 and TAC2. These results were validated by the analyses on the ADEPT cohort which present similar characteristics in terms of severity and inflammatory clusters as U-BIOPRED (2) and Table S7. Figure S2 shows a heat map of the macrophage module ES according to sputum granulocytes. In summary, these data highlighted the relative enrichment of most modules in paucigranulocytic and healthy volunteers with the same modules enriched in neutrophilic (modules 8, 27, 30-31 and 49) (all  $P < .001$ ) and eosinophilic (modules 15, 21 and 29) (all  $P \leq .03$ )





**FIGURE 3** Heat map of enrichment scores (ES) for the 49 macrophage activation modules according to transcriptomic-associated cluster (TAC1, TAC2 or TAC3). GC, glucocorticoids; HDL, high-density lipoprotein; IFN- $\gamma$ , interferon  $\gamma$ ; IL-10, interleukin 10; IL-13, interleukin 13; IL-4, interleukin 4; LA, lauric acid; LiA, linoleic acid; LPS, bacterial lipopolysaccharide; OA, oleic acid; PA, palmitic acid; PGE2, prostaglandin E2; SA, stearic acid; TNF- $\alpha$ , tumour necrosis factor; TPP, TNF- $\alpha$  + prostaglandin E2 + Toll-like receptor 2 ligand. Where no value is given, the results were not significant

asthma as described using TAC analysis in the U-BIOPRED cohort. The same modules as in TAC3 were decreased in ES in the ADEPT paucigranulocytic group (modules 8, 15, 27, 29, 30 and 31).

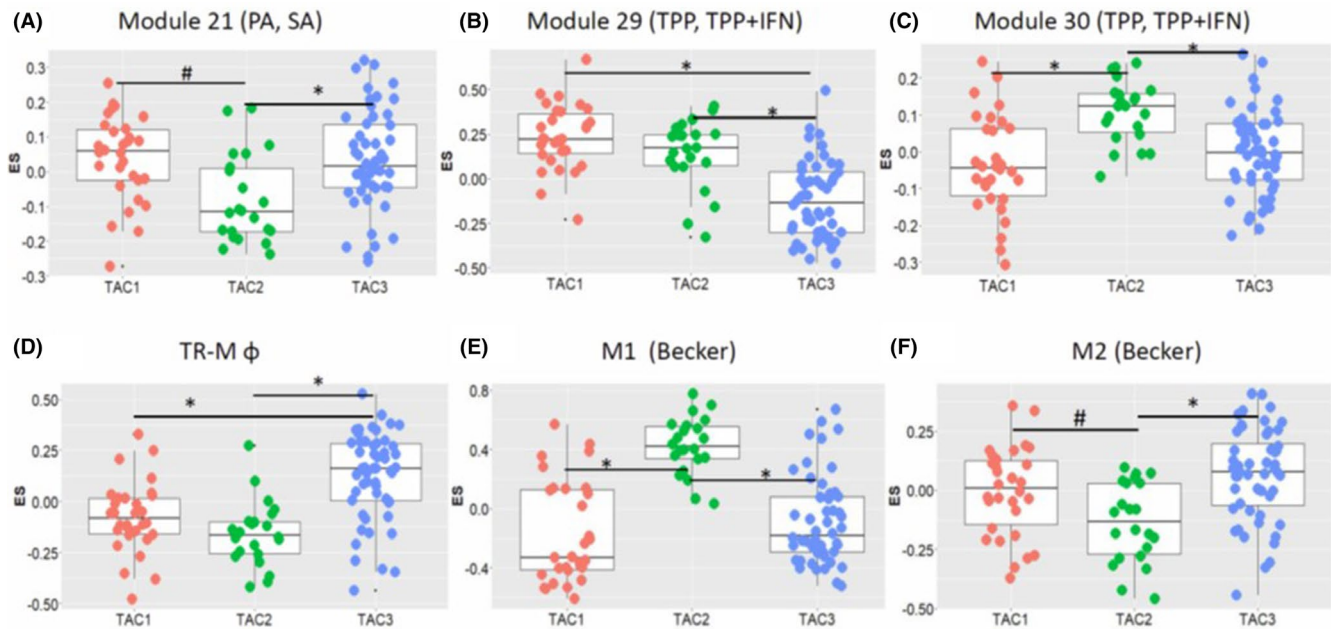
### 3.3 | Macrophages signatures in airflow obstruction

The same modules 15, 21 and 29 that were overexpressed in severe asthma and associated with inflammation driven by innate immunity were correlated with airflow obstruction (Figure S3). In contrast, most M1- and

M2-related modules were inversely associated with obstruction. The TR-M $\phi$  signature did not correlate with airflow obstruction (Figure S4).

### 3.4 | Drivers of macrophage activation according to severity and TAC characterization

A number of different macrophage signatures were enriched in severe asthma and according to TAC categorization. Examination of the specific mediators associated with each macrophage module



**FIGURE 4** Representative analysis of selected macrophage module enrichment scores (ES) according to transcriptomic clusters (TACs). Gene set variation analysis (GSVA) plots of individual subject ES for (A) module 21—PA- and SA-stimulated, (B) module 29—TPP- and TPP + IFN-stimulated and (C) module 30—TPP- and TPP + IFN-stimulated. The ES for tissue-resident macrophages (D, TR-M $\phi$ ), classically activated macrophages (E, M1 from Becker) and alternatively activated macrophages (F, M2 from Becker) are also shown. HV, healthy volunteers; IFN- $\gamma$ , interferon  $\gamma$ ; MMA, mild/moderate asthma; PA, palmitic acid; SA, stearic acid; SAns, severe asthma nonsmokers; SAsm, severe asthma smokers and ex-smokers; TPP, TNF- $\alpha$  + prostaglandin E2 + Toll-like receptor 2 ligand (Pam3CSK4). \* $P < .001$ ; # $P < .05$ . Where no value is given, the results were not significant

according to TAC does not provide clear evidence for the critical factors that may underpin macrophage activation in each TAC (Figure S5). We therefore pooled the activated genes within each module<sup>9</sup> for severe asthma and for each TAC and examined the pathways and gene ontology terms associated with these groups to determine whether specific pathways or driver mechanisms were present.

Combining the macrophage gene signatures enriched in severe asthma and performing PPI network analysis identified several pathways important in severe asthma including innate immunity, leucocyte activation, neutrophil degranulation, IL-4/IL-13 signalling, TLR and IL-17 signalling and leukotriene synthesis (Table 3, Figure 5). The pathways associated with macrophages activated in TAC1 patients highlighted cholesterol and steroid biosynthesis pathways along with pathways associated with Th2 activation and defence responses (Figure S6, Table S8). In addition, the PPI networks identified as being preferentially activated in TAC2 patients included immune and inflammatory pathways linked to inflammasome and neutrophil activation (Figure S7, Table S9). Finally, TAC3-associated PPI pathways reflect metabolic and mitochondrial function in addition to cytokine signalling and viral response pathways (Figure S8, Table S10). These data expand the pathways associated with TACs described previously and highlight the importance of macrophages in severe asthma pathophysiology.

## 4 | DISCUSSION

Sputum macrophage numbers are reduced in severe asthma compared to mild-moderate asthma and healthy volunteers. GSVA analysis of macrophage signatures demonstrated decreased ES in severe asthma compared to mild-moderate asthma and healthy volunteers except for CI-M $\phi$  activated by FA and an M2-like module. Module enrichment occurs in purified AM populations validating the use of signatures to give additional insight compared with that obtained using the classic M1 and M2 classification. Glucocorticoid/high-density lipoprotein-exposed and M1-like macrophages were enriched in the severe asthma smokers vs severe asthma nonsmokers. Sputum macrophage numbers are significantly decreased in TAC1 compared to TAC2 and TAC3 groups and the ESs for most signatures were greater in TAC3 compared to TAC1 and TAC2 subjects. TR-M $\phi$  were enriched in TAC3 and associated with mitochondrial function. Overall, macrophage activation is attenuated in severe granulocytic asthma except for enrichment of specific macrophage subsets indicative of distinct inflammatory pathways.

NF- $\kappa$ B activation is common to both modules 15 and 29 suggesting its activation in severe TAC1 and TAC2 asthmatics. NF- $\kappa$ B regulates inflammatory and innate immune gene expression<sup>18</sup> during asthma pathogenesis.<sup>19</sup> Module 15 (an M2 subtype) is also associated with TLR pathway activation. Most TLRs are expressed on

macrophages and their activation induces inflammatory cytokines involved in the recruitment of T cells (CCL3 and CCL4), monocytes (CCL1) and neutrophils (CXCL1 and CXCL8).<sup>20-23</sup> Module 29 is associated with chronic inflammation and TNF pathway activation.<sup>9</sup> Macrophages possess several TNF receptors including TNFR1 and 2, TL1A, OX40, 4-1BB, CD30, CD40, LIGHTR, LT $\beta$ R, GITR, BAFF, APRIL and TWEAK which all stimulate NF- $\kappa$ B.<sup>24,25</sup> In addition, OX40 stimulation promotes T-cell proliferation and survival<sup>26,27</sup> by the regulation of IL-2 production.<sup>28</sup>

The third module enriched in severe asthma is module 21 that is associated with eicosanoids biosynthesis via the 5-LOX pathway. These macrophages are stimulated by palmitic acid, a pro-inflammatory factor for monocytes and associated with increased mitochondrial ROS production.<sup>29</sup> CysLT promotes Th2 polarization, activation of eosinophils (confirmed by the high ES in TAC1) and collagen production by fibrocytes<sup>30</sup> which could explain the association with airflow limitation found in the present study.

The most significant difference between severe asthma smokers and severe asthma nonsmokers in our analysis is the high enrichment of module 14 in smoking severe asthmatics, which is involved in phago-lysosome fusion and lymphocyte migration. In mice, nicotine inhibits phagosome formation in infected macrophages and induces the production of TGF- $\beta$  by regulatory T cells.<sup>31</sup>

Modules 15, 21 and 29 were enriched in both severe asthma and TAC1 subjects reflecting those with severe eosinophilic T2 asthma. Module 15 represents a subset or state of M2 macrophages stimulated by IL-4/IL-13 which secrete chemokines including CCL18 and CCL24 (eotaxin 2) recognized to promote eosinophil infiltration.<sup>3,4</sup> According to the specific stimuli, M2 macrophages are divided in 4 subpopulations: M2a induced by IL4/IL13 which activate Th2 responses; M2b stimulated by LPS or IL-1R ligand with an immunoregulator effect and favoured by Th2 response, increasing the production of IL-10; M2c prompted by glucocorticoids or IL-10 involved in the inflammation resolution and tissue remodelling (production of TGF $\beta$ ).<sup>4,7</sup>

Even though the M2a state seems to be particularly involved in severe asthma and TAC1, this does not preclude a downstream cascade linking with M2b activation. This may explain the association of the module 15 with the Toll-like receptor signalling pathway. In contrast with TAC1, all subsets of M2 macrophages are enriched in TAC3 and involved in metabolic processes (modules 10-14 and 45), homeostasis (modules 47-48), inflammatory resolution by stimulating phagocytosis, antigen processing and immunoregulation (modules 14 and 46). These results reflect the complex activation state of sputum macrophages in asthma in response to distinct stimuli and their high transcriptional and functional diversity.

TAC2 subjects were enriched for macrophage modules involved in pathogen responses including M1 macrophages. IFN- $\gamma$ - and LPS-stimulated M1 macrophages enable phagocytosis of viral/bacterial antigens by cross-presentation via MHC class I,<sup>32</sup> inflammasome and TNF- $\alpha$  activation<sup>24,33</sup> and neutrophilic airway inflammation.<sup>34</sup> Other modules up-regulated in TAC2 indicate the importance of Th17 (modules 15 and 20) and NK cells (module 32).<sup>33,35,36</sup> Taken together, these data highlight the heterogeneity of sputum macrophages and that modules identify greater differences than consensus M1 and M2 signatures.

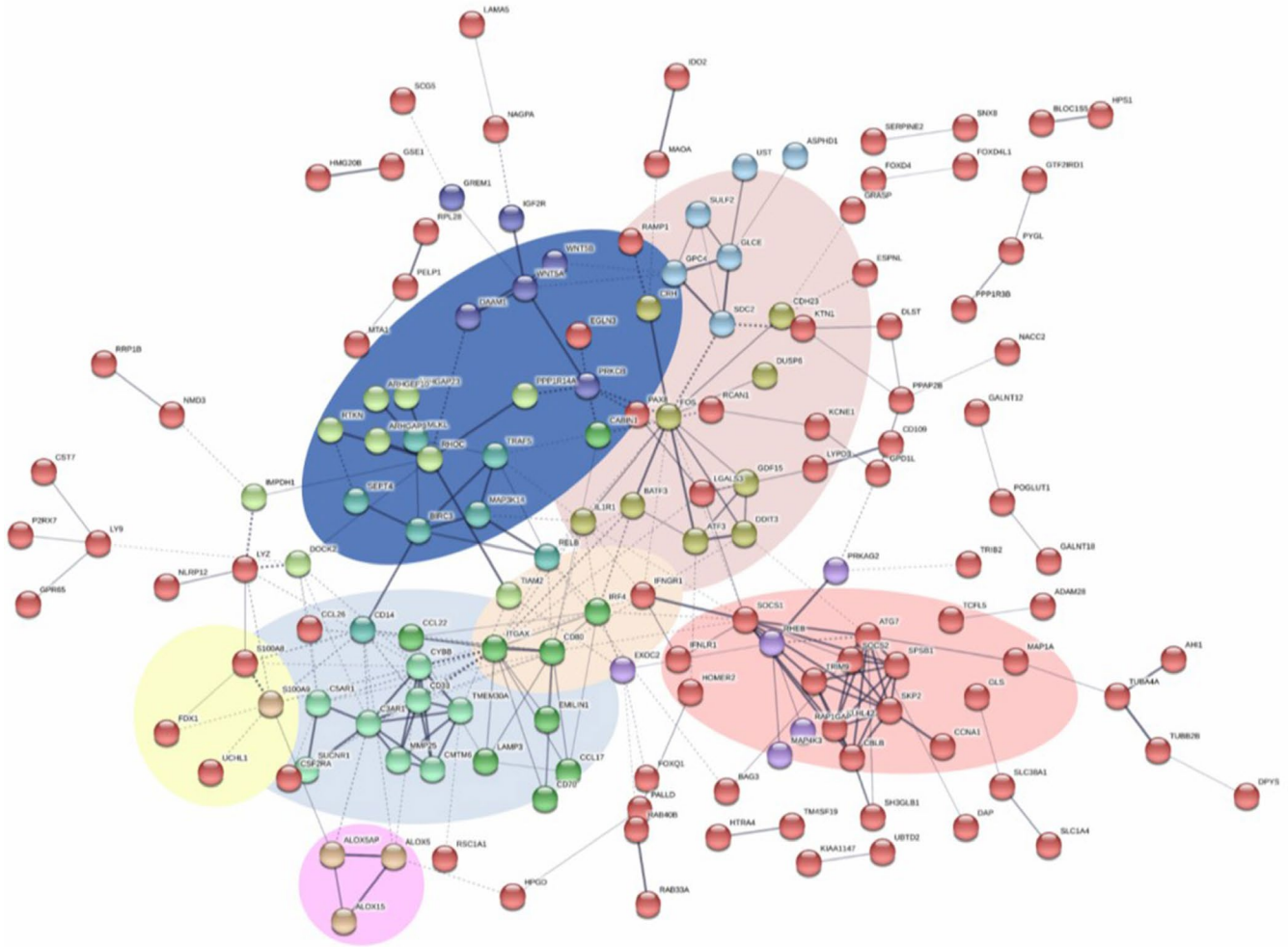
Data concerning the role of TR-M $\phi$  in severe asthma are lacking. We show no significant change in TR-M $\phi$  enrichment according to severity but elevated enrichment in TAC3 subjects. Most of the genes within the TR-M $\phi$  signature are involved in mitochondrial function (TOMM20, RAC1, NDUFA4, CAD, SIRPA, APOA1BP), lipid metabolism, tissue homeostasis and apoptosis but some play roles in anti-infectious defence via TNF- $\alpha$  (TNF), TLR (CD36) and inflammasome (SIGLEC1) pathways.<sup>37</sup> During early inflammation, TR-M $\phi$  could switch from M2 (associated with OXPHOS) to M1 polarization with enhanced glycolysis to rapidly obtain the energy needed for migration, ROS and pro-inflammatory cytokines production (via NF- $\kappa$ B activation) and phagocytosis.<sup>29</sup> Conversely, the resolution of inflammation occurs through the interaction of oxidized

**TABLE 3** Pathways involved in macrophage activation in severe asthma compared to mild-moderate asthma and healthy controls using PPI network analysis (String db)

Pathway	ID	FDR P value
Innate immune system	Reactome: HSA-168249	<.001
Regulation of cellular response to stimulus	GO: 0048583	<.001
Inflammatory response	GO: 0006954	<.001
Leucocyte activation involved in immune response	GO: 0002366	<.001
Neutrophil degranulation	Reactome: HSA-6798695	<.001
Interleukin-4 and interleukin-13 signalling	Reactome: HSA-6785807	<.001
NF- $\kappa$ B signalling	KEGG:04064	.004
Toll-like receptor cascades	Reactome: HSA-168898	.015
IL-17 signalling	KEGG: 04657	.016
Synthesis of leukotrienes	Reactome: HSA-2142691	.028

Abbreviation: FDR, false discovery rate.





**FIGURE 5** Protein-protein interaction (PPI) network analysis of module genes significantly enriched in macrophages activated in severe asthma compared to mild-moderate asthma and healthy controls. Pink: arachidonic acid metabolism; light blue: leucocyte activation and neutrophil degranulation; green: IL-4 and IL-13 signalling; dark blue: NF- $\kappa$ B signalling; light brown: cellular response to stimuli; red: immune system; and yellow: Toll-like receptor cascades. All genes in each module that was differentially expressed were pooled and used in this analysis

phosphatidylserine from apoptotic membranes and CD36 with to elicit mitochondrial biogenesis, arginase expression and return to an M2 phenotype.<sup>29</sup> The dynamic differentiation of macrophages in response to environmental signals is highly dependent on NF- $\kappa$ B activation and mitochondrial function with increased expression of numerous pro-inflammatory gene characteristic of M1 during the early phase of inflammation while the M2 subtype, predominant in the later phase of inflammation, have reduced inflammatory capacity.<sup>4</sup>

The heterogeneous population of airway macrophages are the primary cell type to encounter airborne pathogens.<sup>38</sup> Our data indicate that in mild-moderate asthma these cells are in a state of preparedness despite ongoing treatment with inhaled corticosteroids but that is not the case in patients with severe disease. Previous evidence has highlighted a defect in BAL macrophage and monocyte-derived macrophage phagocytosis in severe asthma.<sup>39-41</sup> This has been reported as being associated with enhanced expression of PGE<sub>2</sub>,<sup>39</sup> reduced IL-10 expression<sup>42</sup> or the persistent presence of viral infection.<sup>43</sup>

The importance of macrophages in mediating asthmatic inflammation is becoming increasingly clear.<sup>38</sup> In mice, pharmacological suppression of alternatively activated YM1 + M2 macrophages using the galactin-3 pathway inhibitor cynaropicrin resulted in reduced eosinophilic lung inflammation and less collagen deposition around airways and a shift towards neutrophilic inflammation and worse lung function.<sup>44</sup> These data revealed an important role for M2 macrophages generally in airway remodelling and the role of specific M2-like macrophages associated in airway remodelling and AHR should be further studied.

This is the first study using gene signatures to extensively analyse the enrichment and activation of macrophage subtypes in asthma according to severity, molecular phenotype and the presence of airflow limitation. To the best of our knowledge, it is the first time that the enrichment of TR-M $\phi$  has been analysed in asthma where it plays a more important role in the paucigranulocytic inflammatory phenotype. Another strength of this study is the relatively large data set, the validation in another severe asthma cohort (ADEPT)

and the relative accessibility of the sputum compartment for future comparative analysis. Nevertheless, there are several limitations to the study including the failure to obtain functional data from these macrophage modules and the need to isolate or define these macrophage subtypes using another approach such as cell sorting. It is possible that the results may reflect, at least in part, the varying proportional make-up of the immune cells present across the various subgroup comparisons, and future studies should use single cell sequencing or other approaches to address this. Furthermore, analysis of BAL and tissue macrophages may provide additional insights in the pathogenesis of severe asthma.

In summary, macrophage numbers and activation status are generally decreased in severe asthma suggesting an innate immune defect in this population. Enrichment was found only in three modules involved in the regulation of inflammatory response by TLR/TNF/NF- $\kappa$ B activation, IL-2 production and leukotriene biosynthesis. M1 macrophages were highly expressed in TAC2 and correlate with sputum neutrophilia, inflammasome, IFN, TNF and TLR pathways in contrast to M2 which were more highly expressed in TAC1 and TAC3 and correlated with metabolic process and eicosanoids biosynthesis. TR-M $\phi$  were predominantly associated with paucigranulocytic asthma. Understanding the functional diversity of macrophages and the modulation of their phenotypes in asthma might provide us a great opportunity for designing novel and more effective therapeutic strategies.

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#### CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

#### AUTHOR CONTRIBUTIONS

AT, NZK, YB and SP made substantial contributions to the acquisition and analysis of the data, and PH, YG, KFC and IMA made substantial contributions to the conception and interpretation of the work. AT and IMA drafted the initial manuscript, and all authors provided substantial input into the revision and interpretation of the manuscript. All authors approved the final version for submission and accept responsibility for the accuracy and integrity of the work.

#### DATA AVAILABILITY STATEMENT

The transcriptomic data are deposited in the GEO (Gene Expression Omnibus) database ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) with accession number GSE76262.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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