Markers Involved in Innate Immunity and Neutrophil Activation are Elevated during Acute Human Anaphylaxis: Validation of a Microarray Study

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Keywords

Allergy · Anaphylaxis · Myeloid cells · Neutrophils

Abstract

Background: We have previously identified the upregulation of the innate immune response, neutrophil activation, and apoptosis during anaphylaxis using a microarray approach. This study aimed to validate the differential gene expression and investigate protein concentrations of “hub genes” and upstream regulators during anaphylaxis. **Methods:** Samples were collected from patients with anaphylaxis on their arrival at the emergency department, and after 1 and 3 h. mRNA levels of 11 genes (*interleukin-6* [IL-6], *IL-10*, *oncostatin M* [OSM], *S100A8*, *S100A9*, *matrix metalloproteinase 9* [MMP9], *FASL*, *toll-like receptor 4* [TLR4], *MYD88*, *triggering receptor expressed on myeloid cells 1* [TREM1], and *cluster*

of differentiation 64 [CD64]) were measured in peripheral blood leucocytes using qPCR. Serum protein concentrations were measured by ELISA or cytometric bead array for 6 of these candidates. **Results:** Of 69 anaphylaxis patients enrolled, 36 (52%) had severe reactions, and 38 (55%) were female. Increases in both mRNA and protein of IL-10, S100A9, MMP9, and TREM1 were observed. OSM, S100A8, TLR4, and CD64 were upregulated and IL-6 protein concentrations were increased during anaphylaxis. Both FASL and soluble Fas ligand decreased during anaphylaxis. **Conclusion:** These results provide evidence for the involvement of innate immune pathways and myeloid cells during human anaphylaxis, validating previous microarray findings. Elevated S100A8, S100A9, TLR4, and TREM1 expression, and increased S100A9 and soluble TREM1 protein concentrations strongly suggest that neutrophils are activated during acute anaphylaxis.

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Introduction

Allergies are a global health concern, with increases in the numbers of adverse reactions to foods in children and drugs in adults [1]. The rapid progression from the onset of allergic symptoms through to a potentially life-threatening reaction affecting multiple organ systems is a hallmark of anaphylaxis [2]. The immune mechanisms driving human anaphylaxis are poorly understood. However, an IgE-mediated pathway, involving the activation of mast cells, has been the predominant focus of clinical investigation [3]. During this process, the initial exposure to an allergen stimulates the production of allergen-specific IgE, which bind to IgE receptors on the surface of mast cells or basophils. On a subsequent exposure, allergens bind to these antibodies, causing IgE receptor cross-linking, which initiates degranulation [4]. This releases an array of mediators including histamine and mast cell tryptase that contribute to the physiological signs of anaphylaxis such as vasodilation and bronchoconstriction [5, 6]. Much still remains unknown about how the localized mast cell signal becomes rapidly amplified into potentially lethal systemic immune activation.

A previous microarray study performed in our laboratory aimed to investigate differential gene expression during the course of anaphylaxis [7]. This study investigated 6 patients with moderate anaphylaxis who were sampled over the first 3 hours after arrival at the emergency department. An increasing number of genes were differentially expressed over this time, culminating in a storm of genetic activation. Several key pathways including inflammation, innate immunity, apoptosis, neutrophil activation, and chemotaxis were upregulated, suggesting these pathways play central roles in the anaphylactic response [7]. The present study aimed to investigate some of these processes more closely during anaphylaxis by analyzing the mRNA expression of some important genes in peripheral blood leucocytes and their corresponding protein levels in serum/plasma, where possible.

The selected targets were identified in our previous microarray study [7] as either “hub genes” or upstream regulators for the pathways of interest including: interleukin (IL)-6; IL-10; oncostatin M (OSM); S100 calcium-binding proteins A8 (S100A8) and A9 (S100A9); matrix metalloproteinase 9 (MMP9); Fas ligand (FasL); toll-like receptor 4 (TLR4); myeloid differentiation primary response 88 (MyD88); triggering receptor expressed on myeloid cells 1 (TREM1); and cluster of differentiation 64 (CD64). IL-6, IL-10, and OSM are cytokines that play important roles in innate immunity by performing either

pro- or anti-inflammatory functions [8, 9]. S100A8 and S100A9 are proteins contained within phagocytes that are released following cellular activation or damage [10]. They are known as danger-associated molecular patterns and the binding of extracellular S100A8/S100A9 to certain activating surface receptors including TREM1 and TLR4 (which associates with the intracellular adaptor protein MyD88) amplifies inflammation [11]. MMP9 is released from myeloid cells, primarily neutrophils, following their activation/degranulation [12]. FasL is a surface protein that binds to Fas receptor and stimulates apoptosis of susceptible Fas-expressing cells [13]. However, once cleaved by matrix metalloproteinases, soluble FasL competes with surface FasL for Fas binding and does not trigger apoptosis. Therefore, while surface FasL is proinflammatory, high concentrations of soluble FasL promote cell survival [14]. Finally, CD64 is the high-affinity IgG receptor present on the surface of macrophages, monocytes, and neutrophils. Anaphylaxis via an IgG-mediated pathway has been demonstrated in experimental mouse models but is not well studied in humans [15].

Investigation into the changes in mRNA expression and protein concentrations of this panel of targets would provide insight into the cell types and pathways activated during anaphylaxis and validate the results of our previous work.

Materials and Methods

Patient Recruitment

Study participants and healthy controls were enrolled in our prospective, observational Critical Illness and Shock Study (CISS) between September 2010 and January 2014 in 4 Australian Emergency Departments. The methodology for CISS has been previously described [16]. Patients enrolled in the study meet a case definition of critical illness, which excludes mild (skin-only) allergic reactions, and then undergo serial blood sampling and clinical data collection at protocolled time points. The time points for this study were as soon as practicable after enrolment criteria were identified (T0), and 1 h (T1) and 3 h after enrolment (T3). These time points reflect those from the microarray study. At each time point, 1 × 3.5 mL serum separating tube and 2 × 2.5 mL Blood RNA PAXgene™ tubes (PreAnalytiX GmbH, Switzerland) were collected. Serum was collected, aliquoted, and stored immediately at –80 °C until analysis. RNA PAXgene™ tubes were collected and placed immediately at 4 °C, then transferred to –20 °C within 72 h, before final storage at –80 °C until analysis.

Ethics Approval and Consent

Ethics approval was obtained from the Human Research Ethics Committees at each hospital (Royal Perth Hospital, Fremantle Hospital, Armadale-Kelmscott Memorial Hospital: EC 2009/080; Austin Hospital: H2012/04477). Since the need for emergency care

took priority, waiver of initial consent was approved under the provision of paragraph 2.3.6 of the National Health and Medical Research Council Ethical Conduct guidelines (2007). Once the study could be explained to them, fully informed written consent was obtained. Patients were given the option of declining further involvement and allowing the destruction of samples collected up to that point.

Patient Cohort

We selected cases that satisfied a clinical definition of anaphylaxis based on National Institute of Allergy and Infectious Diseases/Food Allergy and Anaphylaxis Network (NIAID/FAAN) criteria [2]. To test for differences associated with severity, cases were reviewed by 3 physician investigators (S.G.A.B., D.M.F., and G.A.) and classified into 2 severity groups, moderate or severe anaphylaxis, based on clinical features according to established criteria [17]. Both moderate and severe anaphylaxis involves multiple organ systems, while the presence of hypoxemia ($\text{SpO}_2 \leq 92\%$ or cyanosis), hypotension (systolic blood pressure <90 mm Hg), and/or neurological compromise (confusion, collapse, loss of consciousness, or incontinence) classifies a reaction as severe. This classification was undertaken separately and blinded to the laboratory analyses. Many of the patients included in this study were also sampled for a separate study investigating neutrophil activation during anaphylaxis [18].

RNA Extraction

RNA was extracted using PAXgene™ Blood RNA Extraction Kits (PreAnalytiX GmbH, Switzerland) by automation with a QIAcube instrument (Qiagen, USA). The purity and integrity of the RNA samples were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and a 2100 Bioanalyzer (Agilent, USA). Samples with RNA integrity number (RIN) values <7 and total RNA <1 μg were excluded. As a result, the quality of included samples was very high (median [interquartile range] 260/280 absorbance ratio: 2.04 (0.09); and median (interquartile range) RIN: 8.7 (0.9).

Quantitative PCR

Complementary DNA (cDNA) was synthesized using SuperScript™ III reverse transcriptase according to the manufacturer's protocol (Thermo Fisher Scientific, USA) and stored immediately at -20°C . Quantitative PCRs (qPCRs) were performed in a total volume of 10 μL , comprising 37.5 ng of each primer (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000492301), 0.5 μL of LightCycler® 480 Reso-Light Dye (Roche Diagnostics, USA), and 1 μL of 10 $\times$ PCR buffer, 5 mM MgCl_2 , 0.2 mM dNTPs, 0.33 Units Platinum® Taq DNA Polymerase (all from Thermo Fisher Scientific, USA), and 2 μL of 1/10 cDNA. A Rotor-Gene™ 6000 (Corbett, Australia) was used to optimize annealing temperatures and magnesium concentrations for each set of primers. Triplicate reactions were set up in 384 well plates and a ViiA™ 7 Real-Time PCR System (Applied Biosystems, USA) was used with the following cycling conditions: 50°C for 2 min; 95°C for 10 min; and followed by 40 cycles of 95°C for 15 s, annealing temperature (online suppl. Table S1) for 15 sec, and 72°C for 15 s. Single product amplification was confirmed using dissociation curves established by increasing the temperature of the samples from 60 to 95°C stepwise at 0.05 $^\circ\text{C}$ per second.

Template Cloning for Standard Curve Preparation

RNA extracted from PBLs stimulated overnight with phorbol myristate acetate (Sigma-Aldrich, Australia) was used to prepare cDNA. Targets of interest were amplified using the same primers used for qPCR and products were ligated into pGEM®-T Easy vectors (Promega, Australia). JM109 competent cells (Promega, Australia) were transformed with the construct. Ampicillin-resistant colonies were grown in liquid culture and plasmid DNA was prepared using the QIAprep Spin Miniprep Kit (Qiagen, Australia). Cloned sequences were verified on both strands using Sanger sequencing by the Australian Genome Research Facility. Plasmids were linearized with AatII (New England Biolabs, USA) and standard curves were prepared.

Analysis using qBase Plus

ViiA7™ software (Thermo Fisher Scientific, USA) determined Cq values using the Baseline Threshold algorithm. Three reference genes, *RPS18*, *HPRT*, and *YWHAZ* were determined as appropriate to normalize Cq data using qBase+ software, version 2.6 (Biogazelle, Belgium). Replicates that varied by greater than 0.8 Cq were excluded.

Measuring Protein Concentrations

Serum concentrations of IL-6, IL-10, and soluble FasL were measured by cytometric bead array flex sets (BD Biosciences, USA) as described previously [5]. Samples were diluted 1:4 in assay diluent, measured in duplicate using a FACSCanto™ II flow cytometer (BD Biosciences, USA), and analyzed using FCAP Array™ version 3.0 software (Soft Flow, Hungary). The lower limits of detection were 1.13 pg/mL IL-6; 0.092 pg/mL IL-10; and 1.838 pg/mL FasL, as defined by the manufacturer. Serum concentrations of MMP9, TREM1, and S100A9 were measured using ELISA (DuoSet® R&D Systems, USA) according to the manufacturer's instructions. The lower limits of detection were 22.06 pg/mL MMP9; 33.15 pg/mL TREM1; and 22.06 pg/mL S100A9. Intra- and inter-assay CVs for the ELISAs were 1.8 and 9.3% for MMP9, 4.9 and 13.0% for TREM1, and 2.9 and 18.5% for S100A9. The protein concentrations of the remaining targets were not measured due to limited sample availability, lack of expression in serum, or duplication in the case of S100A8/S100A9.

Statistical Analysis

Due to small sample sizes, differences in mRNA expression between groups were assessed using nonparametric tests. Where protein targets had concentrations below the assay's detection limits, differences in the proportion of detectable samples in each group were assessed using Fisher's exact tests. The Holm-Bonferroni step-down method was used to adjust the critical value to account for multiple comparisons. When groups had $>50\%$ samples detectable, results were log-transformed to normalize the distribution of data. Tobit regression models were used to assess differences in protein concentrations without excluding the censored data. In targets where all samples had detectable protein concentrations, linear regression models were used to investigate differences between groups. Both Tobit and linear regression models were bootstrapped to produce *p* values that were robust to departures from model assumptions. All statistical analyses were performed using Stata version 12.1 (StataCorp, USA).

Table 1. Patient demographics and clinical observations

	Moderate anaphylaxis	Severe anaphylaxis	All
Number	33	36	69
Age, years, mean $\pm$ SD	36 $\pm$ 15.4	44 $\pm$ 15.6	40 $\pm$ 15.9
Gender, male, <i>n</i> (%)	15 (45)	16 (44)	31 (45)
Cause, <i>n</i> (%)			
Drug	7 (21)	19 (53)	26 (38)
Food	18 (55)	5 (14)	23 (33)
Insect	3 (9)	3 (8)	6 (9)
Physical (including food + exercise)	2 (6)	3 (8)	5 (7)
Other/unknown	3 (9)	6 (17)	9 (13)
Onset to enrolment, mins, median (IQR)	75 (47)	72 (64)	75 (50)
Symptoms, <i>n</i> (%)			
Any skin feature	32 (97)	36 (100)	68 (99)
Any GI feature	11 (33)	18 (50)	29 (42)
Any respiratory feature	32 (97)	29 (81)	61 (88)
Hypoxemia	–	5 (14)	5 (7)
Cyanosis	–	10 (28)	10 (14)
Any cardiovascular feature	7 (21)	35 (97)	42 (61)
Hypotension	–	26 (72)	26 (38)
LOC/Collapse	–	12 (33)	12 (17)
Treatments, <i>n</i> (%)			
Adrenaline [^]	29 (88)	36 (100)	65 (94)
Pre-hospital	8 (24)	18 (50)	26 (38)
Steroids	19 (58)	27 (75)	46 (67)
Fluids	16 (48)	27 (75)	43 (62)
Positive histamine* (>1.2 ng/mL)	13 (52)	21 (60)	34 (57)
Positive MCT** (>11.4 ng/mL, or Δ MCT >2 ng/mL)	16 (52)	32 (89)	48 (72)

[^] Adrenaline administered at any stage.

Note, due to sample availability:

* Histamine was measured by ELISA in 25 moderate and 35 severe patients at enrolment.

** MCT was measured by ImmunoCAP[†] in 31 moderate and 36 severe patients at enrolment.

GI, gastrointestinal; IQR, interquartile range; LOC, loss of consciousness; MCT, mast cell tryptase; Δ MCT, difference between highest and lowest observed MCT concentrations. Skin features = erythema, urticaria, periorbital oedema, angioedema. GI features = nausea, vomiting, abdominal/pelvic pain, incontinence. Respiratory features = dyspnoea, stridor, wheeze, chest/throat tightness, hypoxemia, cyanosis. Cardiovascular features = dizziness, diaphoresis, confusion, hypotension, LOC, collapse.

Results

Cohort Characteristics

This study cohort consisted of 69 patients with a confirmed diagnosis of anaphylaxis, with 36 (52%) of them having severe reactions. The patient demographics and clinical characteristics for each severity group are presented in Table 1. The majority of patients were female, accounting for 38 (55%) reactions. The average age was 40 years, ranging from 16–85 years. Both food and drug triggers each accounted for roughly one third of reactions, with most moderate reactions being food-induced and most severe reactions being drug-induced. The median time between the onset of symptoms and the first blood sample was 75 min, ranging from 10 to 215 min.

Differences Evident at Enrolment (T0)

The raw mRNA expression data is presented in Figure 1, with the p-values presented in Table 2. At T0, the expressions of *IL10* and *OSM* were different between the 3 groups (healthy controls, moderate, and severe anaphylaxis; $p \leq 0.002$). The expression of *IL10* and *OSM* was lower in those with moderate anaphylaxis than that in healthy controls ($p < 0.016$). In contrast, the *OSM* expression was higher in those with severe anaphylaxis than that of healthy controls ($p = 0.045$), and higher in those with severe than moderate anaphylaxis ($p < 0.001$). *IL-10* expression was also higher in those with severe than moderate anaphylaxis ($p < 0.001$), but no difference was found between those with severe anaphylaxis and healthy controls. After adjustment for multiple comparisons, there

Fig. 1. Gene expression during anaphylaxis. Quantitative PCR was performed on mRNA extracted from peripheral blood leukocytes of moderate (MOD, $n = 11$) and severe (SEV, $n = 15$) anaphylaxis patients at the time of enrolment in the emergency department (ED) (T0), and again after one (T1) and 3 (T3) hours. Gene expression data are presented as calibrated normalized relative quantities

(CNRQ). Boxplots identify the median, upper and lower quartiles, and the range. Dotted lines indicate the median CNRQ of healthy controls ($n = 20$). Due to the number and complexity of comparisons, significant differences between groups are described in the results text and in Table 2.

Table 2. *p* values for mRNA comparisons between reaction severities at each time point

	T0				T1				T3			
	all 3 groups	HC vs. MOD	HC vs. SEV	MOD vs. SEV	all 3 groups	HC vs. MOD	HC vs. SEV	MOD vs. SEV	all 3 groups	HC vs. MOD	HC vs. SEV	MOD vs. SEV
IL-6	0.1974				0.4526				0.4649			
IL-10	0.0021	0.0029	0.1789	0.0003	0.0010	0.4835	0.0003	0.0012	0.0008	0.0568	<0.0001	0.0478
OSM	0.0022	0.0159	0.0448	0.0002	0.0147	0.4175	0.0028	0.0126	0.0010	0.0082	0.0002	0.2470
S100A8	0.2716				0.0002	0.0453	<0.0001	0.0226	0.0001	<0.0001	<0.0001	0.2610
S100A9	0.4993				0.0001	0.0061	<0.0001	0.0341	0.0001	<0.0001	<0.0001	0.3095
MMP9	0.0079	0.0009	0.1276	0.0248	0.0002	0.0662	<0.0001	0.0187	0.0001	0.0004	<0.0001	0.3205
FASL	0.0418	0.0076	0.0616	0.1555	0.1014				0.0005	0.0003	0.0013	0.2354
TLR4	0.0664				0.0038	0.0906	0.0004	0.0540	0.0001	0.0014	<0.0001	0.3362
0.05/2	0.0593				0.6318				0.0143	0.0187	0.0038	0.3828
TREM1	0.0251	0.0086	0.0167	0.3361	0.3538				0.0012	0.0027	0.0005	0.4579
CD64	0.7653				0.0181	0.4143	0.0059	0.0089	0.0001	0.0181	<0.0001	0.0104

Bolded *p* values remained significant after application of Bonferroni-Holm step-down correction to adjust for multiple comparisons. Pairwise comparisons were performed when the Kruskal Wallis *p* value for testing across all 3 groups was <0.05. HC, healthy control; MOD, moderate; SEV, severe.

Table 3. Proportion of samples with detectable protein concentrations

Marker	Healthy control, <i>n</i> (%)	Moderate, <i>n</i> (%)			Severe, <i>n</i> (%)		
		T0	T1	T3	T0	T1	T3
IL-6	0/20 (0)	9/33 (30)	15/32 (47)	9/30 (30)	21/36 (70)	25/36 (69)	17/35 (49)
IL-10	0/20 (0)	3/33 (9)	6/32 (19)	6/30 (20)	10/36 (28)	15/36 (42)	11/35 (31)
S100A9	0/20 (0)	9/23 (43)	7/23 (30)	7/17 (41)	12/30 (57)	16/30 (53)	11/26 (42)
MMP9	20/20 (100)	31/31 (100)	29/29 (100)	28/28 (100)	36/36 (100)	36/36 (100)	34/34 (100)
FasL	18/20 (90)	18/33 (55)	19/32 (59)	13/30 (43)	24/36 (67)	25/36 (69)	26/35 (74)
TREM1	20/20 (100)	30/30 (100)	28/28 (100)	26/26 (100)	31/32 (97)	34/34 (100)	33/33 (100)

were no differences in expression found between the 3 groups for *IL6*, *S100A8*, *S100A9*, *MMP9*, *FASL*, *TLR4*, *MYD88*, *TREM1* or *CD64* (Fig. 1, Table 2).

The number of samples with measureable concentrations for each protein has been summarized in Table 3, and *p* values for comparisons are presented in Table 4, both according to group and time point. At T0, only MMP9 was detectable in all samples. The proportion of samples with detectable IL-6 and S100A9 concentrations were different across the 3 groups ($p \leq 0.001$). The proportion of samples with detectable IL-6 concentrations increased significantly with anaphylaxis and again with reaction severity ($p \leq 0.015$). S100A9 was detectable in more samples from anaphylaxis patients than healthy controls ($p \leq 0.002$), with no difference between reaction severities ($p = 1.000$). After adjustment for multiple comparisons, there were no differences found in the proportion of detectable samples be-

tween groups for IL10, soluble FasL, and soluble TREM1 (Table 4). For the proteins detected in >50% of samples (MMP9, soluble TREM1, and soluble FasL), there were no differences in protein concentrations found between healthy controls, moderate, and severe anaphylaxis at T0 ($p = 0.078$, $p = 0.082$, $p = 0.099$ respectively; Fig. 2).

An examination into differences due to reaction trigger (food vs. non-food) identified a significant difference in only 1 protein (soluble TREM1 was lower in food anaphylaxis at T0 and T1, online suppl. Material, see www.karger.com/doi/10.1159/000492301).

Differences Evident after 1 h (T1)

At T1, expression of *IL10*, *S100A8*, *S100A9*, and *MMP9* were different across the 3 groups (healthy controls, moderate, and severe anaphylaxis; $p \leq 0.001$; Fig. 1). Of those target genes, only *S100A8* and *S100A9* had differential ex-

Table 4. values for comparisons of proportion of samples with detectable protein concentration

	T0				T1				T3			
	all 3 groups	HC vs. MOD	HC vs. SEV	MOD vs. SEV	all 3 groups	HC vs. MOD	HC vs. SEV	MOD vs. SEV	all 3 groups	HC vs. MOD	HC vs. SEV	MOD vs. SEV
IL-6	<0.001	0.010	<0.001	0.015	<0.001	<0.001	<0.001	0.084	<0.001	0.007	<0.001	0.204
IL-10	0.008	0.282	0.010	0.066	0.001	0.071	<0.001	0.065	0.011	0.069	0.004	0.398
S100A9	0.001	0.002	0.001	1.000	<0.001	0.010	<0.001	0.162	0.001	0.002	0.001	1.000
MMP9	N/A				N/A				N/A			
FASL	0.025	0.014	0.063	0.333	0.048	0.027	0.106	0.451	0.001	0.001	0.293	0.021
TREM1	1.000				N/A				N/A			

Bolded *p* values remained significant after application of Bonferroni-Holm step-down correction to adjust for multiple comparisons. Pairwise comparisons were performed when the Fisher's exact *p* value for testing across all 3 groups was <0.05. HC, healthy control; MOD, moderate; NA, not applicable (all samples detectable); SEV, severe.

Fig. 2. Protein concentrations during anaphylaxis. **(a)** MMP9 and **(b)** soluble TREM1 in serum from moderate ($n = 33$) and severe ($n = 36$) anaphylaxis patients at the time of admission to the emergency department (ED; T0), and again after one (T1) and 3 (T3) hours. Boxplots identify the median, upper and lower quartiles, and the range. Dotted lines indicate the median concentration of healthy controls ($n = 20$): 304.1 ng/mL MMP9; and 182.3 pg/mL TREM1. Due to the number and complexity of comparisons, significant differences between groups are described in the results text.

pression evident in moderate anaphylaxis compared with healthy controls, and both were elevated ($p = 0.045$ and $p = 0.006$, respectively). However, all four were elevated in severe anaphylaxis compared with both healthy controls ($p < 0.001$) and moderate anaphylaxis ($p \leq 0.034$). After adjustment for multiple comparisons, there were no differences found between the 3 groups at T1 in expression of *IL6*, *OSM*, *FASL*, *TLR4*, *MYD88*, *TREM1*, and *CD64* ($p \geq 0.004$).

Differences in the proportion of detectable samples between healthy controls, moderate, and severe anaphylaxis at T1 were found for *IL-6*, *IL10*, and *S100A9* ($p \leq 0.001$; Table 3, 4). At T1, *IL-6* and *S100A9* were detectable in a higher proportion of anaphylaxis patients than healthy controls ($p \leq 0.010$), regardless of severity. *IL-10* was detectable in a larger proportion of severe patients than healthy controls ($p < 0.001$). After adjustment for multiple comparisons, there were no differences found between groups for soluble *FasL* at T1 ($p = 0.048$). *MMP9* concentrations were 1.8-fold (95% CI 1.3–2.5) higher in moderate anaphylaxis than healthy controls ($p < 0.001$; Fig. 2a). Severe anaphylaxis patients had 3.1-fold (95% CI 2.2–4.4) higher *MMP9* concentrations than healthy controls ($p < 0.001$), and 1.7-fold (95% CI 1.2–2.5) higher than moderate anaphylaxis ($p = 0.003$; Fig. 2a). We found no difference in soluble *TREM1* concentrations between both moderate and severe anaphylaxis patients and healthy controls at T1 ($p \geq 0.123$; Fig. 2b). However, soluble *TREM1* concentrations were 1.3-fold (95% CI 1.05–1.7) higher in severe anaphylaxis than moderate anaphylaxis ($p = 0.018$; Fig. 2b). No differences in soluble *FasL* concentrations were found between healthy controls, moderate, and severe anaphylaxis ($p \geq 0.260$).

Differences Evident after 3 h (T3)

By T3, all target genes except *IL6* and *MYD88* were differentially expressed between the 3 groups, after adjustment for multiple comparisons (Fig. 1, Table 2). Expression was higher in moderate anaphylaxis than healthy controls for *OSM*, *S100A8*, *S100A9*, *MMP9*, *TLR4*, *TREM1*, and *CD64* ($p \leq 0.018$). These 7 targets and *IL10* ($p \leq 0.001$) also had elevated expression in severe anaphylaxis patients compared to that in healthy controls. *CD64* had elevated expression in severe compared to moderate anaphylaxis patients ($p = 0.010$). In contrast, *FASL* expression was reduced in both moderate and severe anaphylaxis patients compared to healthy controls ($p \leq 0.001$ for both comparisons), with no difference between reaction severities ($p = 0.235$).

At T3, we found differences in the proportion of detectable samples between the 3 groups for IL-6, *S100A9*, and soluble FasL ($p \leq 0.001$; Table 3, 4). Both IL-6 and *S100A9* were detectable in a higher proportion of moderate and severe anaphylaxis patients than healthy controls ($p \leq 0.007$). Soluble FasL was detectable in a smaller proportion of moderate patients than either healthy controls or severe patients ($p \leq 0.021$). *MMP9* concentrations were 3.5-fold (95% CI 2.6–4.7) higher in moderate anaphylaxis and 4.0-fold (95% CI 2.9–5.4) higher in severe anaphylaxis than controls ($p < 0.001$ for both tests), but not different from each other ($p = 0.377$; Fig. 2a). At T3, soluble *TREM1* concentrations in severe anaphylaxis patients were 1.4-fold (95% CI 1.1–1.7) higher than those in controls ($p = 0.005$). There was no difference between moderate anaphylaxis and controls, or between reaction severity groups ($p = 0.205$ and $p = 0.168$, respectively; Fig. 2b). We found no difference in soluble FasL concentrations between healthy controls and severe anaphylaxis at T3 ($p = 0.854$). We were unable to investigate other differences in concentrations as <50% of samples were detectable.

Discussion

As a validation of results of a previous microarray investigation by our group [7], this study provides evidence of association with anaphylaxis in nearly all of the selected mRNA and/or protein targets. Taken together, the results provide support for the hypothesis that innate immune pathways and myeloid cells such as neutrophils are activated during human anaphylaxis. The clinical relevance of this innate immune activation remains unclear and future studies are required.

The cytokine results validate the microarray analysis and support previously reported findings in a different cohort of anaphylaxis patients [7, 19]. The increases observed in these cytokines during anaphylaxis highlight the involvement of innate immune cell activation. IL-6 and its family member OSM are proinflammatory cytokines, while IL-10 is an anti-inflammatory cytokine. Elevated IL-6 concentrations are evident in allergic asthma patients [20], a condition that shares many similarities with anaphylaxis. OSM concentrations were also elevated in asthmatics, following allergen but not saline challenge [21]. They are released from a number of cell types including mast cells, macrophages, and neutrophils [8, 9]. Due to the proinflammatory functions of IL-6 and OSM, their release during a reaction likely contributes to the symptoms of anaphylaxis. In contrast, IL-10 has been found to reduce anaphylaxis following the challenge of sensitized mice [22–24]. The upregulation and release of IL-10 evident in our cohort likely represents a counter-regulatory response to restore homeostasis. The severity of a reaction could be, in part, determined by the relative concentrations of the pro- and anti-inflammatory cytokines released, with IL-6/OSM dominance resulting in poorer outcomes and IL-10 dominance improving symptom resolution.

During anaphylaxis, both *S100A8* and *S100A9* were upregulated and with increasing extent as the reactions progressed and resolved, validating the microarray data [7]. There was a marked increase in *S100A9* protein in patients, and by association, the same is likely true for *S100A8* [25]. Activated neutrophils export large amounts of *S100A8* and *S100A9*, both separately and as the heterodimer calprotectin. Although other phagocytes contain and export these molecules, neutrophils are considered to be their primary producers [25]. Therefore, the results of this study are strongly suggestive of neutrophil activation, supporting previous findings from our laboratory [18]. Once exported, these proteins function as danger-associated molecular patterns that amplify inflammatory responses via binding to *TREM1* and *TLR4* receptors on innate immune cells [26, 27]. Ligand binding to *TREM1* triggers the cell to express additional activation receptors, thereby amplifying inflammatory responses [28, 29]. *TLR4* is a pattern recognition receptor that signals cell activation in conjunction with its adaptor protein *MyD88* [30, 31]. Our observations of elevated *TREM1* and *TLR4*, coupled with upregulation of *S100A8* and *S100A9*, suggest this signaling pathway as a mechanism for amplifying innate immune signals during anaphylaxis.

Membrane-bound TREM1 receptors are cleaved by matrix metalloproteinases (MMPs) [32], and both soluble TREM1 and MMP9 concentrations were increased during anaphylaxis. Soluble TREM1 can reduce inflammation by binding to TREM1 ligands in the serum, thereby preventing them from binding to membrane-bound TREM1 [32]. MMP9 is an enzyme produced by leukocytes that degrades the extracellular matrix and basement membrane [33]. Increases in MMP9 have been reported following exacerbation of allergic asthma and in patients with allergic bronchopulmonary aspergillosis (a hypersensitivity reaction to fungus) [34, 35]. In addition, elevated serum MMP9 has been observed following allergen challenge in a mouse model [36]. Elevated MMP9 in anaphylaxis may cause extracellular matrix degradation, allowing inflammatory cells to diffuse into tissues and provoke signs such as erythema and angioedema. However, this increase in MMP9 during anaphylaxis may cause TREM1 shedding, with soluble TREM1 acting as an indirect mechanism for dampening systemic inflammation.

FasL is a membrane-bound ligand for Fas that plays an important role in regulating apoptosis and, in some cases, necrosis [13, 37]. Following shedding from the cell surface by MMPs, soluble FasL can still bind to Fas; however, this no longer results in apoptosis [14]. Therefore, soluble FasL acts as a competitive inhibitor of membrane-bound FasL, and so minimizes apoptosis. The reduction in *FASL* expression over the course of anaphylaxis may reflect an increased translation of transcripts into surface FasL, as immune cells are activated and subsequently prepared for apoptosis. In addition, the reduction in soluble FasL supports a pro-apoptotic environment, which would be necessary to clear inflammatory cells following anaphylaxis.

CD64, also known as FcγRI, is a high-affinity IgG receptor present on the surface of human neutrophils, monocytes, and macrophages [38]. In mouse models, binding of IgG to CD64 induces systemic (IgG-mediated) anaphylaxis, in addition to other inflammatory disorders such as autoimmune arthritis and airway inflammation [38]. The upregulation of *CD64* mRNA reported here is expected to correlate with increased surface expression [39], which would need to be confirmed using flow cytometry or microscopy. Additional CD64 expression on leukocytes increases the likelihood of future reactions occurring via the proposed IgG-mediated anaphylaxis pathway.

The clinical relevance of the innate immune activation observed in this study is currently unclear. Such innate responses could be a bystander response due to the abun-

dance of neutrophils (approximately 60% of peripheral blood leukocytes), and their potential to be mobilized by treatment with adrenaline [40]. Alternatively, these innate immune cells may play a specific role in amplifying initial immune signals, thereby contributing to the systemic physiological symptoms of anaphylaxis. We have observed no evidence to suggest that the innate immune activation prolongs the symptoms of anaphylaxis, or encourages a biphasic reaction. Conversely, their activation may serve to control the reaction and attempt to restore homeostasis through the removal of excess cytokines, mediators, and inappropriately activated cells from the peripheral circulation via phagocytosis.

In conclusion, the findings of this study have provided support for the involvement of the innate immune system during anaphylaxis, particularly the activation of granulocytes such as neutrophils. Products released from neutrophils, such as MMP9 and other potent antimicrobials, can cause non-specific tissue damage. Therefore, the widespread activation of neutrophils may not only amplify the inflammatory signal systemically, but also contribute to the clinical features of anaphylaxis. Further analysis into the surface protein expression of these receptors by flow cytometry or microscopy would improve our understanding of the mechanisms driving receptor production, expression, and cleavage. Additionally, studies investigating the roles of the innate immune cells, such as neutrophils, during anaphylaxis may open up new avenues for improved treatments, prevention, and screening for at-risk individuals.

Limitations

There are limitations to this study that should be acknowledged. First, the assays were performed using whole blood and serum, thus the cellular source(s) of the gene expression signals and protein mediators is unclear. There was a limit to the number of targets we could analyze due to the amount of sample collected from patients. Also, as these patients were recruited under an observational study, no restrictions were made on the treatment delivered. While at enrolment most patients are untreated, it is impossible to be certain whether the changes observed at later time points are influenced by the treatment administered. Future mechanistic studies will assist in further exploring the role/s of these targets. Lastly, while comparisons to healthy controls may not be ideal in the setting of anaphylaxis, the design of the CISS protocol only sampled patients until discharge, and therefore we were unable to analyse changes in these targets relative to each patient's individual baseline.

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Disclosure Statement

The authors declare that they have no conflicts of interest to disclose.

Author Contributions

All authors reviewed and approved the final manuscript. A.F. contributed to the study concept and design, performed the laboratory experiments, contributed to data analysis, and drafted the manuscript. E.B. contributed to the data analysis and manuscript preparation. S.F.S. contributed to the study concept and design, and assisted in obtaining funding support. D.M.F., G.A., and S.P.J.M. contributed to the collection of data. S.B. contributed extensive statistical knowledge to assist in data analysis and interpretation. S.G.A.B. contributed to the study concept and design, obtaining funding support, and collection of data.

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