

Research Article

Eosinophilia and reduced STAT3 signaling affect neutrophil cell death in autosomal-dominant Hyper-IgE syndrome

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The autosomal-dominant hyper-IgE syndrome (HIES), caused by mutations in STAT3, is a rare primary immunodeficiency that predisposes to mucocutaneous candidiasis and staphylococcal skin and lung infections. This infection phenotype is suggestive of defects in neutrophils, but data on neutrophil functions in HIES are inconsistent. This study was undertaken to functionally characterize neutrophils in STAT3-deficient HIES patients and to analyze whether the patients' eosinophilia affects the neutrophil phenotype in *S. aureus* infection. Neutrophil functions and cell death kinetics were studied in eight STAT3-deficient patients. Moreover, the response of STAT3-deficient neutrophils to *S. aureus* and the impact of autologous eosinophils on pathogen-induced cell death were analyzed. No

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specific aberrations in neutrophil functions were detected within this cohort. However, the half-life of STAT3-deficient neutrophils *ex vivo* was reduced, which was partially attributable to the presence of eosinophils. Increased *S. aureus*-induced cell lysis, dependent on the staphylococcal virulence controlling accessory gene regulator (*agr*)-locus, was observed in STAT3-deficient neutrophils and upon addition of eosinophils. Accelerated neutrophil cell death kinetics may underlie the reported variability in neutrophil function testing in HIES. Increased *S. aureus*-induced lysis of STAT3-deficient neutrophils might affect pathogen control and contribute to tissue destruction during staphylococcal infections in HIES.

Keywords: HIES · STAT3 · neutrophils · eosinophils · *S. aureus*



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The autosomal-dominant hyper-IgE syndrome (HIES) is a rare primary immunodeficiency caused by dominant-negative mutations in STAT3 [1, 2]. Associated multisystem features include eczema, delayed shedding of primary teeth, bone fractures, typical facial characteristics, craniosynostosis, and vascular abnormalities. Classical laboratory findings are increased serum IgE levels, low percentage of T_H17 cells, reduced memory B cells and blood eosinophilia [3].

STAT3-deficient HIES patients are particularly susceptible to mucocutaneous candidiasis and recurrent skin and respiratory infections with a rather narrow spectrum of pathogens, in particular *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*), and *Haemophilus influenzae* (*H. influenzae*) [3]. Postinfectious pneumatoceles are pathognomonic, but patients also frequently develop bronchiectasis. These lung complications facilitate secondary opportunistic infections and have a major impact on morbidity and mortality in HIES [4]. Therefore, it seems essential to understand both the particular susceptibility to infection as well as the associated increased tissue destruction in HIES.

A major immunological hallmark of STAT3-deficient HIES is the lack of IL-17-producing T_H cells (T_H17) [5, 6]. T_H17 cytokines augment the production of chemokines and antimicrobial peptides, particularly from keratinocytes and bronchial epithelial cells. Thus, the lack of T_H17 may lead to deficient neutrophil recruitment and reduced antimicrobial killing in local infections, thereby offering an explanation for the observed susceptibility to mucocutaneous and tissue infections [7]. Whereas T_H17 immunity has been closely linked to mucocutaneous candidiasis, invasive lung infections with *S. aureus*, like in HIES, are not typical for other primary immunodeficiencies with reduced IL-17 signaling [8]. Furthermore, it was recently shown that STAT3-deficient patients with somatic mosaicism and a normal T_H17 response may still develop mucocutaneous candidiasis as well as pneumonia [9]. These observations imply that the lack of T_H17 alone

is not sufficient to explain the specific susceptibility to staphylococcal lung infections in STAT3-deficient HIES. Defects in neutrophil function have been suggested to underlie the particular susceptibility to staphylococcal abscesses and lung infections and candidiasis [10–12]. In particular, altered chemotaxis has been reported repeatedly [11–15]. However, published data are inconsistent and variations even within the same patient have been observed [14–18]. In a recent study, STAT3-deficient neutrophils were found to migrate into blisters, indicating normal chemotaxis in HIES *in vivo* [9]. Although there might be temporary variations in the chemotactic capacity of STAT3-deficient neutrophils [13], the heterogeneity of findings, and the lack of correlation with *S. aureus* infections or disease severity in individual patients [15] challenges a direct causal relationship between impaired neutrophil chemotaxis and the clinical phenotype in HIES.

Overall, the discrepant data on neutrophil function in autosomal-dominant HIES is puzzling. Studies performed prior to the discovery of STAT3 deficiency as the genetic cause of HIES (2007) may be confounded by inclusion of patients with other causes of hyper-IgE syndrome. Another explanation for the variable results may be methodological differences in neutrophil function analysis. Density gradient centrifugation, the most commonly employed procedure for neutrophil isolation, leads to a minor contamination with eosinophils due to overlapping densities and thus to a mixed granulocyte preparation [19]. As eosinophil numbers are very low in healthy controls, their presence in these granulocyte preparations is usually considered negligible. However, the influence of increased eosinophil numbers in HIES patients has not been assessed. As eosinophils contain an arsenal of toxic substances that are released during incubation with *S. aureus* [20, 21], it is essential to understand their contribution to the clinical phenotype and functional neutrophil testing in HIES.

Here, we report an enhanced *in vitro* neutrophil cell death in STAT3-deficient HIES patients as compared to healthy controls. Our data suggest that both the presence of eosinophils and reduced STAT3 signaling affect neutrophil cell death kinetics and are associated with increased *S. aureus*-induced cell death.

Results

Variable clinical severity despite similar laboratory findings in STAT3-deficient HIES patients

The study cohort comprised six male and two female STAT3-deficient HIES patients between two and thirty years of age at the time of study inclusion (Table 1). Clinical laboratory findings retrieved from the patient charts showed typical immunological alterations found in STAT3-deficient HIES patients [3], such as elevated IgE levels, low or absent T_H17 cells/IL-17 levels, reduced percentages of marginal zone B cells and/or class-switched memory B cells (Table 1). Blood neutrophil numbers were normal in six patients, but were occasionally below normal limits in HIES #1 and in HIES #5. Eosinophilia, i.e. elevated eosinophil counts in blood (> 500 cells/ μ L), was documented in seven patients (Table 1). The investigated patients consistently showed relatively elevated eosinophil numbers in granulocyte preparations, including the patient with normal absolute eosinophil counts in whole blood. An exception was patient #8, who had a very high percentage of eosinophils in the granulocyte fraction (40%) during the initial analysis, but not at a subsequent analysis (2.5%).

The severity of clinical symptoms was highly variable between the patients, even in those with identical mutations (Table 2). All patients had a history of fungal infections. *S. aureus* was isolated from various body sites in seven of eight patients. Available clinical histology results revealed presence of neutrophils in abscesses of three patients and in the bronchoalveolar lavage (BAL) fluid of two patients. Furthermore, eosinophils were present in one BAL fluid sample and in three histological samples (Table 2, Supporting Information Fig. 1).

The patients received symptomatic treatment including antimicrobial prophylaxis, abscess drainage, and IgG-substitution when needed, but also lung surgery in three patients (data not shown).

Basic neutrophil functions were not altered in STAT3-deficient HIES patients

Several neutrophil functions were tested (Table 3). Due to limitations in sample size and availability not all tests could be performed for every patient. Reactive oxygen species (ROS)-release, chemotaxis, and adhesion were normal in the patients tested. Early whole blood and neutrophil-mediated staphylococcal killing was similar between investigated patients and controls. Neutrophils from patients released neutrophil extracellular traps (NETs) upon phorbol 12-myristate 13-acetate (PMA) stimulation (Supplement Movie 1–2, Supporting Information Figs. 2–4). In summary, STAT3-deficient HIES patients in our cohort did not show substantial alterations in basic neutrophil functions.

Cell death kinetics were accelerated in neutrophils from STAT3-deficient HIES patients

Quantification of cell death by flow cytometry (Fig. 1A–C) revealed accelerated cell death of STAT3-deficient neutrophils compared

to concomitantly analyzed control cells (Fig. 1D). In particular, increased early apoptosis (intact cell membrane) (Fig. 1E) was found at four and nine hours after cell isolation and enhanced late apoptosis/necrosis (permeabilized cell membrane) (Fig. 1F) was evident at nine and fourteen hours. Interindividual differences were observed in both patients and controls. The percentage of eosinophils at the time of initial measurement, i.e. four hours after cell isolation, correlated strongly with the percentage of late apoptotic/necrotic neutrophils at four hours ($r = 0.92$; $p = 0.0002$) (Supporting Information Fig. 5). A less significant correlation ($r = 0.65$; $p = 0.0418$) was observed with the percentage of early apoptotic neutrophils at four hours. There was, however, no correlation with the percentage of eosinophils at later time points for any of the cell death categories. Cytokine stimulation with IL-8 increased cell viability throughout the observation time (Fig. 1G, Supporting Information Fig. 6). Still, a substantial difference in cell death remained discernible between patients and controls for both early apoptosis (Fig. 1H) and late apoptosis/necrosis (Fig. 1I) at nine and fourteen hours. Increased cell viability and reduced early apoptosis in combination with maintained differences in cell death between patient and control cells were also found for a limited number of samples treated with G-CSF and GM-CSF (Supporting Information Fig. 7). The enhanced cell death kinetics in HIES patients compared to controls were also observed during visual comparison of granulocyte suspensions by microscopy (Supporting Information Fig. 2C and D, Supporting Information Fig. 8) and during live cell imaging (Supplement Movie 3–6).

S. aureus-induced cell death was increased in STAT3 deficiency and upon addition of eosinophils

S. aureus is a major pathogen in STAT3-deficient HIES. We therefore analyzed the impact of *S. aureus* on neutrophil cell death in vitro. Both granulocyte preparations and highly purified neutrophils from STAT3-deficient patients consistently showed increased spontaneous and *S. aureus*-induced lytic cell death as compared to concomitantly analyzed control cells (Fig. 2A–D). When purified neutrophils were supplemented with 20% eosinophils *S. aureus*-induced cell death increased, both in cells from patients and controls (Fig. 2E). Due to sample size limitations the data shown in Figure 2 could not be pooled for statistics. However, to verify the effect of STAT3 inhibition and eosinophil supplementation on lactate dehydrogenase (LDH) release, the experiments were repeated with cells from healthy donors employing a chemical STAT3 inhibitor (ST18).

In contrast to cells isolated from HIES patients, chemical STAT3 inhibition of control granulocytes or purified neutrophils alone did not substantially affect spontaneous lytic cell death (Fig. 3A and B, Supporting Information Fig. 9C). However, in line with the observations from primary cells from STAT3-deficient patients, ST18-treated cells showed significantly augmented LDH release upon challenge with *S. aureus*. Similar to the observation obtained from patient samples, the supplementary lytic effect imposed by

Table 1. Laboratory findings and demographic details of the study cohort

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Age	11 years	12 years	15 years	16 years	4 years	26 years	30 years	2 years
Gender	Male	Female	Male	Male	Male	Female	Male	Male
Mutation	H332L	R382L	T622I	K340E	R382W	Y657C	R382L	D371.G380del
Localisation of Mutation	DNA-binding domain	DNA-binding domain	SH2 domain	DNA-binding domain	DNA-binding domain	SH2-binding domain	DNA-binding domain	DNA-binding domain
Age at first symptoms	Newborn period	Newborn period	Newborn period	Newborn period	8 weeks	Newborn period	6 months	3 months
First clinical symptom of HIES	Newborn eczema	Newborn eczema	Newborn eczema	Newborn eczema/impetigo	Superinfected eczema	Infected eczema	Skin abscesses	Skin abscesses
Age when HIES was first suspected	7 years	1 year	6 years	5 years	3 years	19 years	2 years	6 months
Which symptom led to investigation for HIES?	Recurrent abscesses and herpes zoster	Recurrent purulent otitis, eczema, cold abscesses	Pleuropneumonia and pneumatocele, elevated IgE	Recurrent pulmonary infections, skin eczema	Recurrent skin infections and otitis	Recurrent abscesses, skin infections	Abscesses	Skin abscess
Medical care in immunological clinic?	Since age 7 years	Since age 7 years	Since age 6 years	Since age 15 years	Since age 3 years	Since age 20 years	Since age 18 years	Since age 6 months
Documented neutrophil numbers	Some normal but also several low values (range 577–1270/ μ L)	Normal	Normal	Normal	Generally normal, some low values (range 740–990/ μ L)	Normal	Normal	Normal
Eosinophil numbers (highest documented value)	352/ μ L	800/ μ L	800/ μ L	790/ μ L	3180/ μ L	2500/ μ L	1400/ μ L	2200/ μ L
Exemplary % eosinophils among granulocytes documented during experiments	19%	23%	10%	19%	27%	17%	20%	40%
IgE (highest documented value)	1040 kU/L	4101 kU/L	17 081 kU/L	9965 kU/L	1997 kU/L	12 640 kU/L	36 000 kU/L	4270 kU/L
IgG, IgA, IgM	Normal	Normal	Not determined	Normal	Normal	Normal	Normal	Normal [41]
Protein specific antibodies	Anti-tetanus after booster: normal	Anti-tetanus after booster: normal	Anti-tetanus, anti-measles and anti-varicella: detectable	Anti-diphtheria and anti-tetanus after booster: absent; anti-mumps: absent	Anti-tetanus after booster: absent	Anti-diphtheria: normal; Anti-tetanus: low	Anti-tetanus: normal	Anti-tetanus: low; anti-haemophilus: low
Anti-pneumococcal antibodies	Partial positive vaccination titres	Partial positive vaccination titres	Partial positive vaccination titres	Low	Not performed	Low	Normal	Normal
TH17 cells/IL-17 formation	Almost absent (0.04% CD3+CD4+IL-17+ cells)	Almost absent (0.01% CD4+CD45RO+IL-17+ cells)	Almost absent (0.07% CD3+CD4+IL-17+ cells)	Low (0.2% CD3+CD4+IL-17+ cells)	Low (0.27% CD4+CD45RO+IL-17+ cells)	Low IL-17 release from PBMCs [35]	Low IL-17 release from PBMCs [35]	LOW (0.5–1% CXCR-CCR4+CCR6+) [41]
B cell differentiation	Slightly low percentage of marginal zone B cells (4.28%). reduced percentage of class-switched memory B cells (2.92%)	Low percentage of marginal zone B cells (3.78%), normal percentage of class-switched memory B cells (5.81%).	Low marginal zone B cells (3.61%), almost complete lack of CD27+IgD+ and class-switched memory B cells	Low percentage of marginal zone B cells (3.95%) and very low class-switched memory B cells (0.96%)	Low numbers of marginal zone B cells (4.03%) and of class-switched CD27+IgD- B cells (2.13%)	Not performed	Not performed	Low levels of marginal zone B cells (2%) and switched memory B cells (0.5–1%) [41]
FOX P3 expression	Normal	Normal	Normal	Normal	Not performed	Not performed	Not performed	Not performed

DNA, deoxyribonucleic acid; FOX P3, forkhead box P3; HIES, autosomal-dominant hyper-IgE syndrome; IL-17, Interleukin-17; MMR, measles, mumps, rubella; SH2, Src Homology 2; [] = literature reference.

Reference values:

- Neutrophil numbers were considered normal if they were > 1500/ μ L.
- Eosinophilia (i.e. elevated blood eosinophil counts) was considered if eosinophil numbers were > 500/ μ L.
- Markers to detect TH17 cells varied. Internal reference values from full-aged, healthy individuals without STAT3 mutations as provided by the performing laboratory are as follows: CD3+CD4+IL-17+ cells: 0.38–3.1%. CD4+CD45RO+IL-17+ cells: 1.1–4.76%. CXCR-CCR4+CCR6+ cells: 3.8–5.6%.

Table 2. Clinical presentation and complications

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Pneumonia	Recurrent obstructive bronchi-tis/pneumonia below the age of 2 years	ARDS due to RSV-pneumonia requiring ventilation at age of 5 months. Pneumonia at age 1, 2, 9, and 14 years	8 pneumonia (at age 4, 6, 7, 9, 10, and 15 years), 2 times with isolation of <i>H. influenzae</i>	6–7 pneumonia/year between age 0–5 years, 1 pneumonia/year since age 5 years, overall over 30 episodes	None	None	1 pneumonia at age 30 years	None
Skin abscesses	recurrent abscesses various locations: armpit, groin, antecubital fossa, buttocks	twice (forehead, forearm)	twice (buttocks, axilla)	six times (knee, eyelids)	superinfected eczema, recurrent paronychia	recurrent, location not documented	once, location not documented	recurrent, first time at age 3 months (<i>S. aureus</i>)
Abscesses of inner organs	Lymph node abscess	None	Lung abscess requiring drainage and subsequent resection of upper lung lobe	Not documented	Lymph node abscess, due to <i>Streptococcus pyogenes</i>	Not documented	Not documented	No
Other abscesses and skin infections	Recurrent paronychia	Abscess of labium	Dental abscess requiring tooth extraction	Not documented	Not documented	Hip joint	Not documented	No
Otitis media	Recurrent, up to > 10/year. Chronic purulent otitis at age 2 years. Mastoiditis due to <i>S. pneumoniae</i> and <i>S. aureus</i> at age 3 years.	Recurrent otitis. Documented colonization with <i>Pseudomonas aeruginosa</i>	Once	Once	5–6 episodes/year at age 2 and 3 years	Yes, recurrent	Not documented	No
Specific viral infections	Severe varicella at age 6 months, once herpes zoster	Varicella infection	H1N1-infection with central pneumonia and <i>P. aeruginosa</i> colonization	No	No	No	No	No
Positive <i>S. aureus</i> findings	Isolated from otorrhea	Isolated from abscesses, recurrent colonization with MRSA	Once documented in bronchoalveolar lavage, skin colonization	Skin colonization	Skin colonization	Documented from skin, nose, throat	Not documented	Isolated from abscesses
Positive <i>S. pneumoniae</i> findings	Not documented	Not documented	Sputum	Once in bronchoalveolar lavage	Lymph node abscess	Yes, recurrent	Not documented	Not documented
Positive fungal findings	Oral candidiasis, nail mycosis	Genital candidiasis, nail mycosis, oral candidiasis	Oral candidiasis	Tinea pedis, nail mycosis	Oral candidiasis	Nails	<i>Aspergillus, Candida</i> and <i>Scedosporium</i> in the lung	Oral candidiasis; <i>Aspergillus</i> in the nasopharynx
Positive <i>Haemophilus influenzae</i> findings	Not documented	Sputum	Twice during pneumonia, once during bronchitis (sputum)	Not documented	Once during otitis	Yes, recurrent	Yes	Once in nasopharynx during acute respiratory infection
Positive <i>Pseudomonas aeruginosa</i> findings	Not documented	Recurrently documented during otitis	Only colonization	Colonization	Not documented	No	Chronic colonization of the lungs	No
Pneumatocele	No	Yes	Yes (left and right lung)	Yes (right lung)	No	No	No	No
Bronchiectasis	No	No	Yes	Yes	No	No	Yes, progressive	No
Clubbed fingers/hippocratic nails	No	No	No	Yes	No	No	No	No

(Continued)

Table 2. Continued

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Bronchoalveolar lavage (BAL)-results (as documented by the diagnostic pathology department)	Not performed	No growth	During pneumonia: numerous neutrophils, lymphocytes and some macrophages, sparse <i>S. aureus</i> , very few eosinophils. Numerous degenerative transformed macrophages and neutrophils.	Prior to lung resection: numerous granulocytes, no bacteria. After lung resection: <i>S. pneumoniae</i> , numerous granulocytes, numerous squamous epithelia.	Not performed	Not performed	Not performed	Not performed
Histology (as documented by the diagnostic pathology department)	Lymph node abscess: granulocytic absceding inflammation with histiocytic reaction	Skin biopsy: inflammatory infiltration of lymphatic and histiocytic cells	Dental abscess: phlegmonous inflammation with presence of numerous neutrophils, some lymphocytes and plasma cells, few eosinophils (Supporting Information Fig. 1A). Lung abscess: purulent, absceding pneumonia with massive numbers of alveolar macrophages, histiocytes, neutrophils, cell detritus. On the edges, fibrinoid connective tissue with overall fewer cells, but several eosinophils, plasma cells and neutrophils. In bronchia: cell detritus, foam cell macrophages and neutrophils (Supporting Information Fig. 1B).	Lung resection (not during acute infection): findings are compatible with multiple past pneumonia. No significant eosinophilia, no granuloma, no acute purulent infection, no fungal infection.	Lymph node aspiration: immature suppurative granuloma reaction. Multiple eosinophils in the granuloma center (Supporting Information Fig. 1C and D).	Not performed	Not performed	Pyogenic granuloma on the tongue

ARDS, acute respiratory distress syndrome; RSV, respiratory syncytial virus.
The data in this table was retrieved from patient charts and/or questionnaire.

Table 3. Assessed neutrophil functions

Patient-ID	HIES #1	HIES #2	HIES #3	HIES #4	HIES #5	HIES #6	HIES #7	HIES #8
Neutrophil chemotaxis	Normal	n.p.	Normal	n.p.	n.p.	n.p.	n.p.	n.p.
Neutrophil adhesion	Normal	n.p.	Normal	n.p.	n.p.	n.p.	n.p.	n.p.
Quantitative NET formation	Normal	Normal	Normal	Normal	Normal	n.p.	n.p.	n.p.
ROS release upon PMA stimulation	Normal	Normal	Normal	n.p.	Normal	n.p.	n.p.	Normal [41]
Whole blood-killing of <i>S. aureus</i>	Normal	Normal	n.p.	n.p.	Normal	Normal	Normal	Normal
Neutrophil-killing of <i>S. aureus</i>	n.p.	Normal	n.p.	n.p.	n.p.	Normal	Normal	Normal

n.p., test not performed; [], literature reference.

eosinophils during staphylococcal infection was also present in healthy controls (Fig. 3A). When evaluating whether presence of eosinophils during granulocyte isolation in healthy controls affected the LDH release, a mild correlation was observed for the percentage of eosinophils within the granulocyte fraction and the LDH release of DMSO-treated granulocytes stimulated with vehicle or *S. aureus* (Supporting Information Fig. 9A and B). The correlation was lost after preincubation with ST18, which led to a significant increase in LDH upon *S. aureus* challenge (Supporting Information Fig. 9C).

The staphylococcal *agr*-system influences cell lysis during STAT3 inhibition

Secreted virulence factors such as hemolysins and leukocidins have been implicated in mediating the lytic effect of *S. aureus* on eosinophils and neutrophils [21, 22]. Therefore, we studied whether the expression of secreted virulence factors was essential for the observed cell lysis in STAT3-inhibited samples. Granulocytes from healthy blood donors were stimulated with either an *agr*⁺ wild type *S. aureus* strain (8325-4 [WT]) or its isogenic *agr*[−] mutant (WA250 [*agr*[−]]) in the presence or absence of STAT3 inhibitor. The *agr*[−] strain is characterized by reduced expression of α -, β -, δ -, and γ -hemolysins, leukocidins, enterotoxins, and TSST-1 [23]. Challenge with the *agr*[−] strain diminished LDH release in both STAT3-inhibited and control samples (Fig. 3B). The highest cell lysis was observed in STAT3-inhibited samples treated with wild type bacteria expressing the *agr* gene regulator.

STAT3 inhibition in granulocytes does not affect the release of IL-1 β , TNF- α , or IL-8 by *S. aureus*

Different cytokines implicated in cell death were analyzed in supernatants of STAT3-inhibited and control granulocytes following incubation with WT and *agr*[−] *S. aureus*. IL-1 β or TNF- α were not detected in any of the samples (three independent experiments with six different controls, detection limit IL-1 β 3.9 pg/mL, TNF- α 15.6 pg/mL). However, IL-8 production was more pronounced when stimulated with the *agr*[−] in comparison to the WT strain (Fig. 3C). Still, IL-8 production did not differ significantly between STAT3-inhibited and control samples.

Discussion

STAT3-deficient HIES patient are strikingly susceptible to acquire staphylococcal infections and suffer from enhanced tissue destruction following pulmonary infections. So far, this specific susceptibility remains incompletely understood. Given the role of neutrophils in the defense against *S. aureus* and *Candida*, a specific dysfunction in STAT3-deficient neutrophils would offer a logical solution [11–13]. However, in agreement with other recent studies we found normal superoxide production [1, 3, 24],

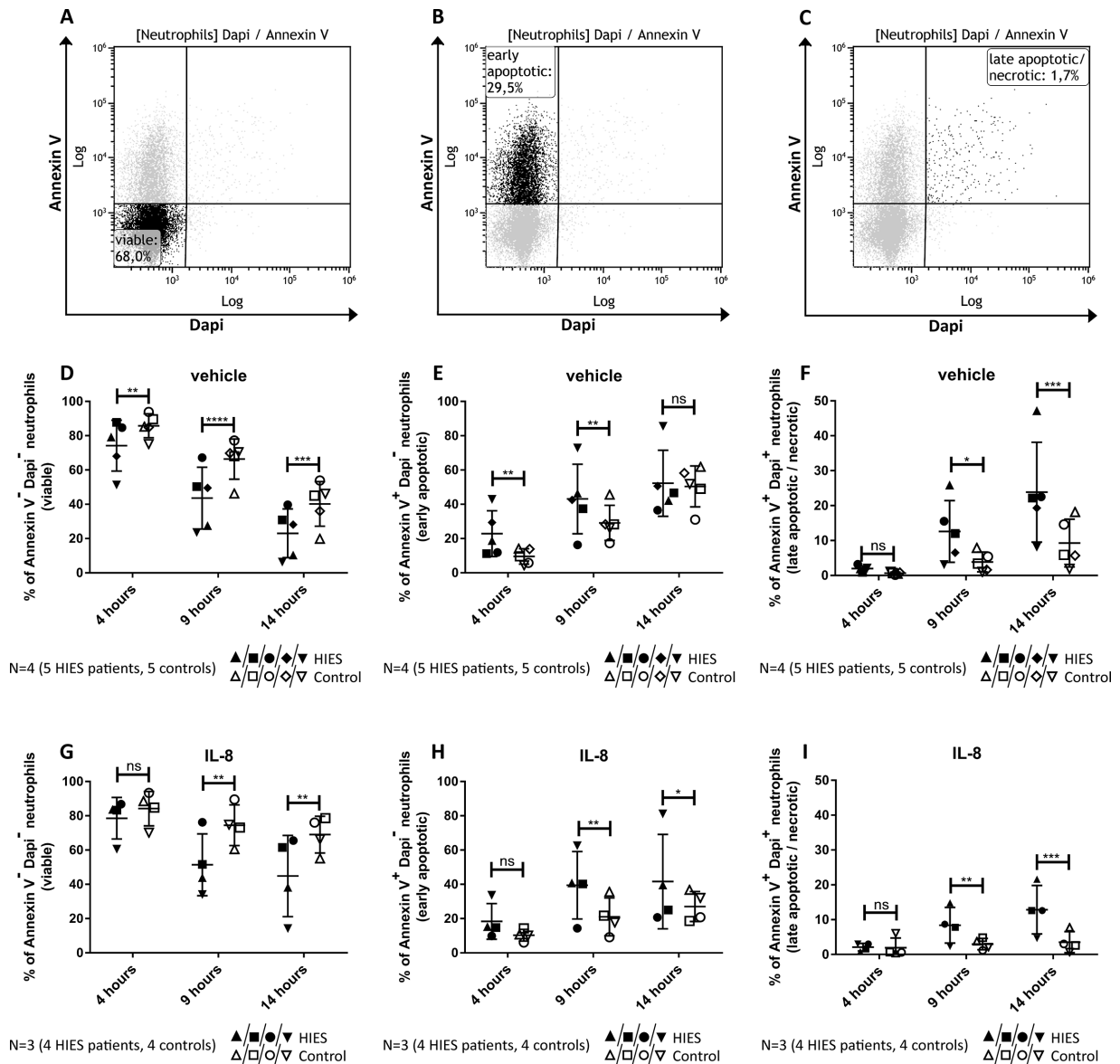


Figure 1. Accelerated cell death kinetics of neutrophils from STAT3-deficient HIES patients. Granulocytes were isolated from STAT3-deficient HIES patients and concomitant control samples followed by cytokine or vehicle stimulation. At designated time points (four, nine, and fourteen hours), equal amounts of the samples were stained with Annexin V and Dapi and analyzed by flow cytometry (detailed gating strategy see Supporting Fig. 11). (A–C) displays the same dataset highlighting the gating strategy for the identification of the following cell death categories according to the respective Annexin V and Dapi staining: (A) viable (Annexin V⁻ Dapi⁺), (B) early apoptotic (Annexin V⁺ Dapi⁺) and (C) late apoptotic/necrotic (Annexin V⁺ Dapi⁻) neutrophils. Primary necrotic (Annexin V⁻ Dapi⁺) neutrophils are not displayed since no significant differences were found. (D–I) Quantitative data showing the percentages of (D, G) viable, (E, H) early apoptotic and (F, I) late apoptotic/necrotic neutrophils after stimulation with (D–F) vehicle or (G–I) IL-8. (D–I) Data are shown as mean ± SD and are pooled from four (D–F) and three (G–I) independent experiments, respectively. Each patient was matched to the concomitant daily control. Matched samples are displayed with the same symbol (dark symbols = HIES patients; light symbols = controls). RM (repeated measures) two-way Anova with Bonferroni-Analysis for multiple comparison was used for statistical analysis ($p > 0.05$ = not significant (ns); $p \leq 0.05$ = *; $p < 0.01$ = **; $p < 0.001$ = ***; $p < 0.0001$ = ****).

chemotaxis [3], and preserved staphylococcal killing [1]. Moreover, tissue samples and BAL fluid from our patients revealed presence of neutrophils during infections, thus underscoring functional chemotaxis, despite low or absent IL-17 signaling. This observation and the maintained infectious phenotype of STAT3-deficient individuals with somatic mosaicism and a normal T_H17

compartment [9] suggest that additional factors are crucial for their susceptibility to infection.

The majority of HIES patients present with eosinophilia, which was also true for seven of our eight patients. However, even in the absence of blood eosinophilia, the percentage of eosinophils among isolated granulocytes was generally elevated in patients.

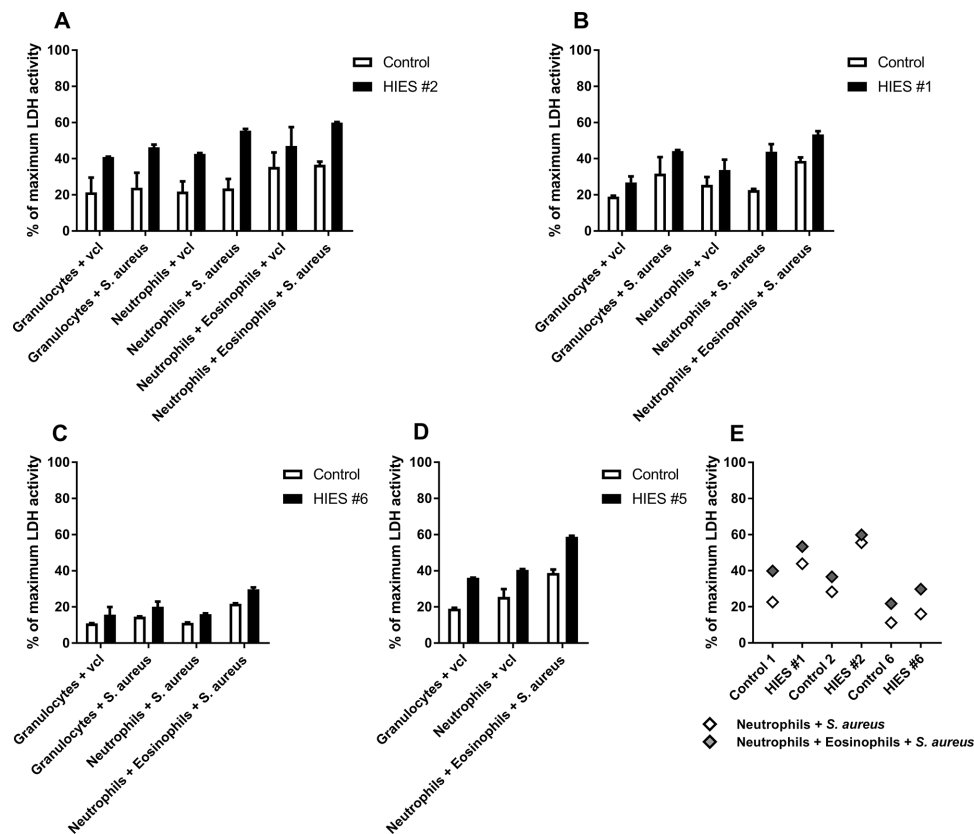


Figure 2. Augmented spontaneous and *S. aureus*-induced lytic cell death in STAT3-deficient HIES patients. Eosinophils and neutrophils were purified by magnetic cell separation from granulocyte suspensions of STAT3-deficient HIES patients and concomitant healthy controls. LDH release was measured in supernatants five hours after costimulation of various granulocyte fractions (original granulocyte suspension, highly purified neutrophils, purified neutrophils + 20% purified eosinophils) with vcl (vehicle = medium) or *S. aureus* WT (MOI 5). Due to limitations in cell availability not all conditions could be performed in every experiment. (A–D) Three individual experiments are shown, including cells from four different HIES patients and their concomitant controls. Depicted is the mean with SD of at least two sample replicates. B+D were performed at the same time point and contain the same controls. (E) The effect of supplementation of neutrophils with eosinophils on *S. aureus*-induced cell lysis for available samples in A–C is shown. Data are pooled from three independent experiments.

Analyzed neutrophil cell death kinetics in vitro were accelerated in HIES patients in the presence of high eosinophil numbers in the granulocyte preparations. This effect was attributable to both enhanced early apoptosis and late apoptosis/necrosis. As the percentage of eosinophils correlated only at initial time points with the percentage of early apoptotic and late apoptotic/necrotic neutrophils, eosinophilia may most likely only partly explain the accelerated cell death kinetics seen in vitro. Because apoptotic neutrophils are functionally impaired in vitro [25], accelerated cell death may become relevant in vivo, particularly during infections when neutrophil survival is prolonged by cytokines. Consistent with our findings, it was previously reported that STAT3-deficient neutrophils showed enhanced proinflammatory cytokine expression, yet, without the expected associated apoptosis delay [1]. In our hands, patient neutrophils responded with delayed apoptosis to IL-8, GM-CSF, and G-CSF. Yet, cell death was still clearly enhanced when compared to control neutrophils, particularly at late time points. The partially maintained anti-apoptotic proper-

ties are probably related to residual STAT3 signaling in affected individuals, as well as alternative signaling pathways [26–28].

Given the relevance of *S. aureus* infections in HIES patients, we assessed how stimulation with *S. aureus* would affect neutrophil fate in the presence of STAT3 deficiency and eosinophils. In the limited number of available samples we observed that granulocytes and purified neutrophils from STAT3-deficient HIES patients showed an increased baseline of spontaneous cell lysis and were more sensitive to *S. aureus*-induced lytic cell death than the respective control cells. Supplementation of purified neutrophils with eosinophils led to higher *S. aureus*-induced lytic cell death in both HIES patients and controls. This suggests that the observed accelerated cell death rate in the context of high levels of eosinophils in vitro may also play a role in staphylococcal infections. In our cohort, eosinophils were documented in three histological samples and one BAL obtained during acute infections. Others have reported on eosinophilic infiltrations in the lung, skin or abscesses in individuals with STAT3-deficient HIES [24, 29]. Interestingly,

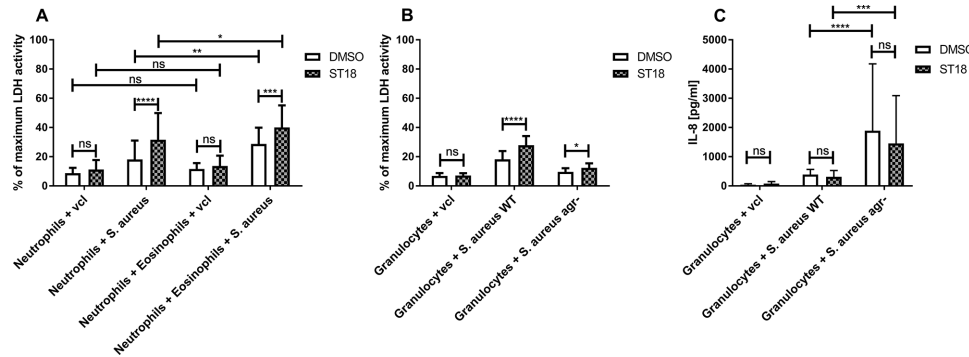


Figure 3. Increased *S. aureus*-induced lytic cell death in STAT3-inhibited cells. Eosinophils and neutrophils were purified by magnetic cell separation from healthy control granulocyte suspensions. The original granulocyte suspension and/or purified neutrophils were incubated for 60 min with 20 μ M ST18 (STAT3 Inhibitor XVIII, BP-1-102) or DMSO. Subsequently, cells were washed and coincubated with vcl (vehicle = medium), *S. aureus* WT and/or *S. aureus* agr⁻ (both MOI 5) for five hours. (A) LDH release after stimulation of purified STAT3-inhibited (ST18) and control (DMSO) neutrophils with/without supplementation of 20% eosinophils with vcl or *S. aureus* WT for five hours is shown. Displayed are pooled results from eight independent experiments with cells from nine different controls. (B) LDH release following stimulation of STAT3-inhibited (ST18) and control (DMSO) granulocytes with vcl, *S. aureus* WT, or *S. aureus* agr⁻ (both MOI 5) for five hours is shown. Displayed are pooled results from four independent experiments with cells from eight different controls. (C) IL-8 release of STAT3-inhibited (ST18) and control (DMSO) granulocytes following stimulation with vcl, *S. aureus* WT, or agr⁻ (both MOI 5) for five hours is shown. Displayed are pooled results from three independent experiments with six different controls (detection limit 3.1 pg/mL). Statistical analysis A–C: Depicted is the mean with SD. RM (repeated measures) two-way Anova with Bonferroni-Analysis for multiple comparison was used for statistical analysis for A–C ($p > 0.05$ = not significant (ns); $p \leq 0.05$ = •; $p < 0.01$ = ••; $p < 0.001$ = •••; $p < 0.0001$ = ••••).

eosinophils appear to be particularly susceptible to cell lysis and degranulation when incubated with *S. aureus* [20, 21]. In our experiments employing eosinophil supplementation to control neutrophils, increased cell lysis became evident when stimulating with *S. aureus*. Therefore, local eosinophilia could potentially limit antimicrobial efficacy of neutrophils during manifest infection.

The use of a chemical STAT3 inhibitor allowed assessing the functional consequences of STAT3 deficiency in the absence of high numbers of eosinophils during sample preparation. While STAT3 inhibition alone did not increase cell lysis, there was a significant augmentation of *S. aureus*-induced lytic cell death in STAT3-inhibited cells. The initial concentration of eosinophils did only mildly correlate with cell lysis of granulocytes and the correlation was completely lost after STAT3-inhibition. This suggests that reduced STAT3 signaling in neutrophils may negatively affect cell survival during staphylococcal infection, independent of eosinophilia.

It has been argued that increased neutrophil lysis by certain *S. aureus* strains favors pathogen survival and augments disease severity in otherwise healthy individuals [30]. The staphylococcal quorum sensing agr-operon activates transcription and production of virulence factors that may destroy neutrophils [22]. We demonstrate that the effect of reduced STAT3 signaling on cell death is most pronounced in the presence of secreted staphylococcal virulence factors present in the agr⁺ wild type strain. Enhanced anti-apoptotic IL-8 induction may partly explain the protection from cell death during incubation with the toxin-deficient agr⁻ mutant. Yet, this does not appear to be relevant for the observed differences in cell death during deficient STAT3 signalling, as IL-8 expression did not differ between STAT3-inhibited and control samples. The precise mechanism underlying the increased *S. aureus*-induced cell

lysis in STAT3 deficiency remains to be elucidated. Nevertheless, our findings may be clinically relevant, as a recent study showed that HIES patients are frequently colonized with highly virulent and antibiotic-resistant *S. aureus* strains [31].

STAT3 is ubiquitously expressed. Thus, the specific infection susceptibility in HIES at mucocutaneous sites, is likely shaped by both immune and epithelial cells. Potential epithelial factors include altered cytokine expression and deficient release of antimicrobial factors [32–34]. Together with impaired IL-17 formation [7, 35] these factors could lead to a higher bacterial load at the infection site. In this setting, STAT3-deficient neutrophils that respond with increased cell lysis to virulent *S. aureus* may further impair infection control and contribute to tissue destruction. Maintained neutrophil functionality in HIES, however, seems to be sufficient to prevent invasive organ and blood stream infections.

Our results also offer an explanation for the controversial literature on neutrophil functions (e.g. chemotaxis) in HIES. Density gradient centrifugation with subsequent erythrocyte lysis is the most widely used method for rapid isolation of neutrophils [30, 36, 37]. Red blood cell lysis with ammonium chloride is beneficial for maintenance of neutrophil properties [37], but may impair eosinophil viability and induce eosinophil degranulation [38, 39]. While this effect may be negligible in donors with low eosinophil contamination, a high eosinophil percentage in granulocyte preparations, as in HIES, negatively affects neutrophil viability and may then alter neutrophil function analysis, particularly if analysis is delayed [40].

Limitations of our study include the restricted number of patients and that not all experiments could be performed for each patient due to limited clinical material. Yet, despite interindividual variability our results were consistent. Moreover, the

effect of eosinophil supplementation and STAT3 inhibition was reproducible in healthy controls. Still, the small sample size impedes correlating experimental findings with disease severity in individual patients. Furthermore, although neutrophils and eosinophils were detected in histological samples from our patients, their interaction *in vivo* may be affected by additional factors, which are not present during *in vitro* testing. It would be particularly interesting to further evaluate how the specific milieu induced in STAT3-deficient lung epithelium may alter the host-pathogen interactions and affect postinfectious tissue remodeling.

In summary, we describe eight unrelated STAT3-deficient HIES patients with variable clinical presentation and overall normal basic neutrophil functions *in vitro*, in spite of staphylococcal infections and mucocutaneous candidiasis. Histological samples in individual patients indicate that neutrophils are recruited to infected tissues, even though IL-17 formation is impaired. Our data suggest that both STAT3 deficiency and the presence of eosinophils negatively affect neutrophil cell death kinetics, which may hamper *in vitro* neutrophil function testing in STAT3-deficient patients. The presence of eosinophils in abscesses from HIES-patients and the observed increased *S. aureus*-induced cell lysis in presence of eosinophils and STAT3 deficiency offer the possibility that this effect may also be relevant during infections. Further investigations are needed to decipher the precise molecular mechanisms behind this observation and to assess whether increased *S. aureus*-induced cell death in the presence of STAT3 deficiency or eosinophilia affects pathogen control and tissue destruction *in vivo*.

Materials and methods

Study cohort and ethics

The study included eight patients with genetically confirmed STAT3-deficient HIES at the Centre of Chronic Immunodeficiency, Freiburg, Germany or at the Karolinska University Hospital Stockholm, Sweden. Ethical approval was obtained by local ethical committees. All patients or their legal guardians gave informed consent for participation and data collection. Clinical data were retrieved from questionnaires filled in by patients or their parents, and by reviewing patient charts. Patients #6 and #7 participated earlier in another study [35]. Details on patient #8 were reported previously [41]. Blood samples from healthy controls were obtained either anonymously from the blood bank, or following informed consent from unaffected relatives, laboratory or hospital staff. For experiments requiring eosinophils, blood samples were also obtained from healthy donors without infectious history, but with supposedly asthmatic or allergic predisposition not requiring current medication, following informed consent. Here, sample blood volume was increased to 80 mL to achieve sufficient eosinophil numbers for experiments.

Granulocyte isolation and purification of neutrophils and eosinophils

Blood samples were processed within three hours after blood draw using a standard protocol for neutrophil isolation [36, 37]. Each patient sample was accompanied by a concomitant control sample. Samples were processed via density gradient centrifugation with isotonic Biocoll with 10 mM hydroxyethyl-piperazineethanesulfonic acid buffer (HEPES) (density of 1.077 g/mL, Biochrom). Red cells were lysed with lysis buffer containing ammonium chloride, potassium hydrogen carbonate, and ethylenediaminetetraacetic acid (EDTA) (pH 7.4) at 4°C, followed by washes in PBS and final suspension in medium, i.e. sterile-filtered RPMI-1640 (without Phenolred, endotoxin tested; Sigma-Aldrich) + 2% human albumin (Albunorm 20%, Octapharma) unless otherwise indicated. Viability and purity of the obtained granulocytes was routinely $\geq 95\%$ as assessed by trypan blue staining and flow cytometry, respectively. In healthy controls, contamination of granulocytes with eosinophils was usually $< 7\%$. For selected experiments, granulocytes were further purified into neutrophils and eosinophils employing the EasySep Human Neutrophil Enrichment kit (Stemcell) and the EasySep Human Eosinophil Enrichment kit (Stemcell), generally achieving a purity $\geq 99\%$ for neutrophils and $\geq 94\%$ for eosinophils (Verification of cell origin see Supporting Information Fig. 10A–F). In order to highlight the differences between the preparations, we use the term *granulocytes* when referring to the initial isolation product and *neutrophils* when further purification steps were employed.

Bacterial strains

We used *S. aureus* strain 8325-4 (WT) and its isogenic *agr*-mutant WA250 (*agr*[−]) with known lower toxin and enzyme expression [23].

Granulocyte cell death kinetics by flow cytometry

Granulocytes were stimulated with medium (vehicle = vcl) or cytokines (IL-8, G-CSF, GM-CSF; PeproTech or R&D Systems). At designated time points, equal cell numbers were stained with Annexin V (BD Bioscience) and 4', 6-diamidino-2-phenylindole dihydrochloride (Dapi, Life Technologies) to detect apoptotic and necrotic cells, respectively. The following categories were employed: early apoptotic (Annexin V⁺ Dapi[−]) and late apoptotic/necrotic (Annexin V⁺ Dapi⁺), primary necrotic (Annexin V[−] Dapi⁺) and viable (Annexin V[−] Dapi[−]) neutrophils. For gating strategy see Supporting Information Fig. 11. Neutrophils were generally distinguished from eosinophils by means of their different autofluorescence, e.g. in FL3 (excitation 488 nm; emission filter 620/30) versus FL4 (excitation 488 nm; emission filter 695/30). Samples were analyzed on a Gallios flow cytometer (Beckman Coulter). No additional antibodies were used during cell death assessment. Cell type origin in the autofluorescent gates

was confirmed in separate experiments (Supporting Information Fig. 10D–F). Flowcytometric data was analyzed with Kaluza software (Beckman Coulter).

Lytic cell death determination by LDH release

In order to assess spontaneous and *S. aureus*-induced lytic cell death, various granulocyte fractions (original granulocyte suspension, purified neutrophils and/or purified neutrophils + 20% eosinophils) were incubated with vehicle (medium), *S. aureus* WT and/or *agr*[−] mutant at a multiplicity of infection (MOI) of five for five hours at 37°C, 5% CO₂. After two hours, gentamicin (final concentration 200 µg/mL) was added to kill off extracellular bacteria. After centrifugation the obtained supernatant was used for analysis of LDH and cytokines. LDH release was measured with the Cytotoxicity Detection Kit^{PLUS} (Roche) according to the manufacturer's instructions.

STAT3 inhibition of neutrophils from healthy controls

Granulocytes or purified neutrophils were incubated for one hour with the STAT3 Inhibitor XVIII, BP-1-102 (Calbiochem) here named ST18, at a concentration of 20 µM (Supporting Information Figs. 12 and 13) or DMSO diluted in medium as vehicle control, followed by two washes with PBS. Subsequently, spontaneous and *S. aureus*-induced lytic cell death of various granulocyte fractions was determined as described above.

Measurement of cytokines

IL-8, IL-1β, and TNF-α were measured in supernatants obtained after five hour incubation of STAT3-inhibited or DMSO-treated control granulocytes with medium (vcl), *S. aureus* WT or *agr*[−] employing commercial ELISA-kits (BD Bioscience OptEIA ELISA kits).

NET quantification and visualization

Granulocytes were seeded in black 96-well plates and stimulated with vehicle or 25 nM phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) at 37°C, 5% CO₂ for 2.5 hours. Then, the cell impermeable DNA staining dye Sytox green (Invitrogen) was added and fluorescence signal was detected with a fluorescent reader. NET formation was visually confirmed by fluorescence microscopy (Supporting Information Fig. 2). In selected experiments, granulocytes were stained with CellTrackerTM dye (Invitrogen) prior to stimulation. Following two hours of stimulation, Sytox green was added and the plate was analyzed with an automated fluorescence microscope (Olympus Scan^R) (Supporting Information Figs. 3 and 4).

Staphylococcal killing assay

Fifty microliters whole blood and/ or 2×10^5 granulocytes in RPMI-1640 + 2% human albumin were incubated with serum-opsonized *S. aureus* (2×10^5 cfu, MOI 1) at 37°C, 5% CO₂, 130 rpm. After 30 and 120 minutes cells were lysed with 0.1% Saponin and bacterial counts were determined via serial dilution plating.

Neutrophil function tests: ROS, chemotaxis, and adhesion

Basic neutrophil function tests were performed as previously described [42]. In brief, PMA-induced release of ROS was determined by lucigenin chemiluminescence assay. For the adhesion assay, Calcein-AM (Life Technologies) stained cells were stimulated with vehicle, Pam3Cys (Invitrogen), n-formyl-L-methionyl-leucine-phenylalanine (fMLP), PMA, or LPS (all Sigma Aldrich) for 30 minutes, then washed and lysed, followed by detection of fluorescence intensity. For chemotaxis, Calcein-AM stained cells were seeded in Fluoroblok-inserts (BD Falcon). Chemotactic substances such as fMLP, platelet activating factor (PAF), and C5a (all Sigma Aldrich) were pipetted onto the plate bottoms and chemotaxis of cells from the upper insert to the bottom was determined by measuring fluorescence intensity over 45 minutes. Due to limitations in the availability and size of patient samples, not all tests could be performed for every patient.

Statistics

Statistical analysis was performed using GraphPad Prism 7. Patient and concomitant control samples and in vitro treated control samples were matched and analyzed with repeated measures (RM) two-way Anova with Bonferroni-Analysis for multiple comparison. Statistical analysis was only performed if $\geq$ three sample sets were available. Correlation was estimated using Pearson correlation.

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Conflict of interest: None.

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- Abbreviations:** agr: accessory gene regulator · BAL: bronchoalveolar lavage · DMSO: dimethyl sulfoxide · HIES: hyper-IgE syndrome · LDH: lactate dehydrogenase · NET: neutrophil extracellular trap · PMA: phorbol 12-myristate 13-acetate · *S. aureus*: *Staphylococcus aureus* · T_H17: T helper 17 cells · vcl: vehicle
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Supporting Information for

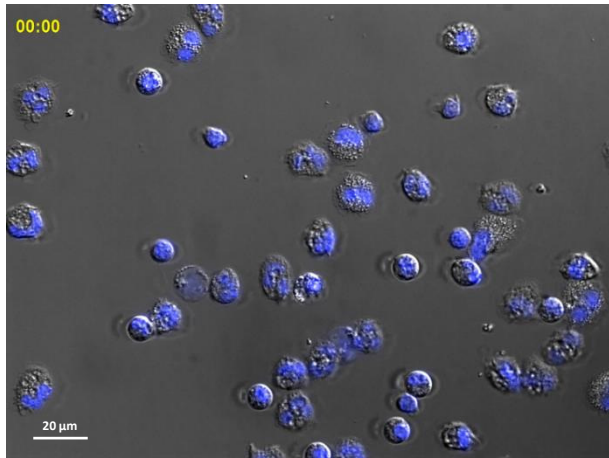
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Susan Farmand, Bernhard Kremer, Monika Häffner, Katrin Pütsep, Peter Bergman, Mikael Sundin, Henrike Ritterbusch, Maximilian Seidl, Marie Follo, Philipp Henneke and Birgitta Henriques-Normark

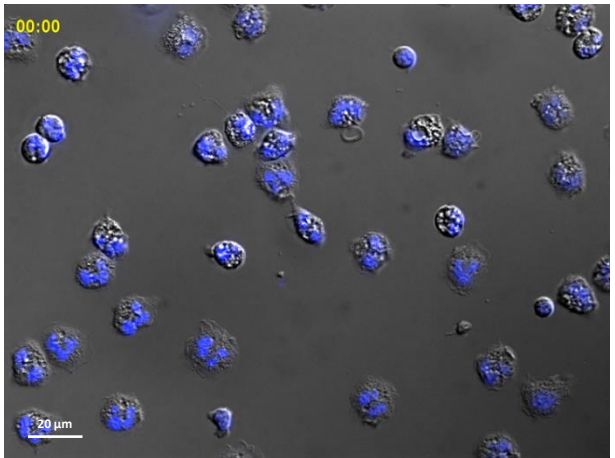
Eosinophilia and reduced STAT3 signaling affect neutrophil cell death in autosomal-dominant Hyper-IgE syndrome

Movies – Supplement

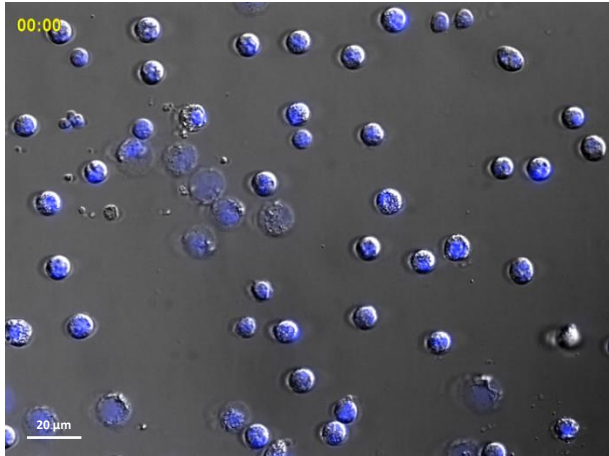
Movie 1: HIES Patient: PMA 25 nM



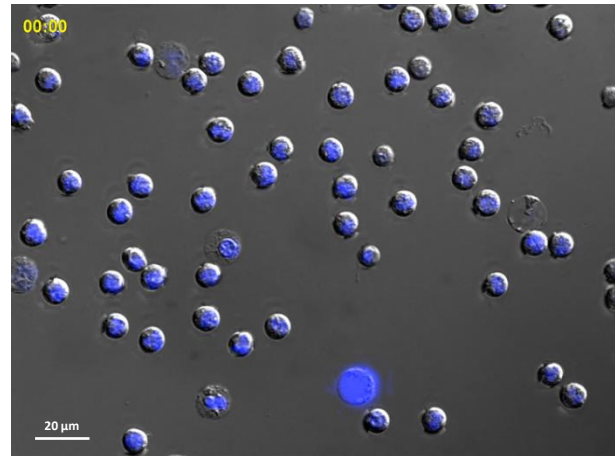
Movie 2: Control: PMA 25 nM



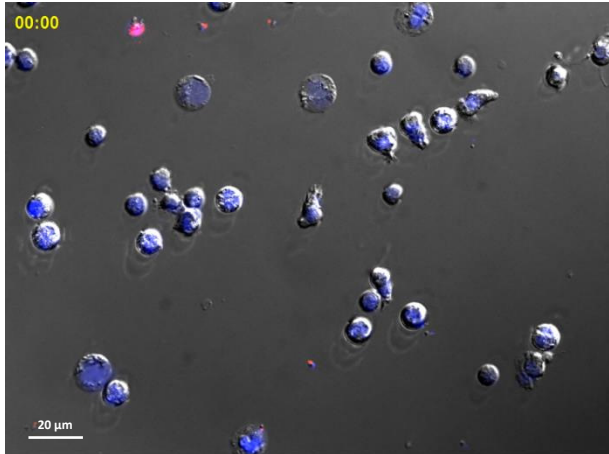
Movie 3: HIES Patient: vehicle



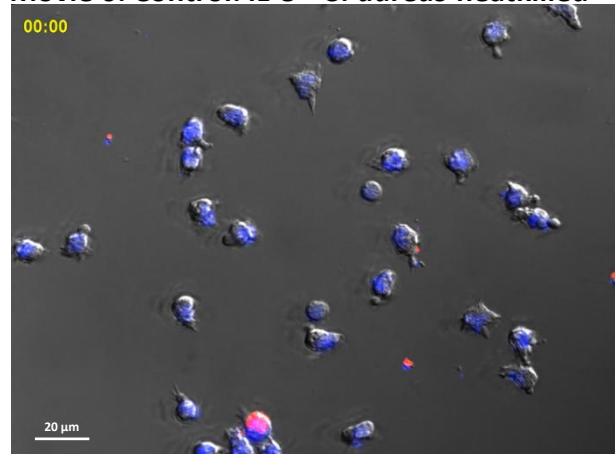
Movie 4: Control: vehicle



Movie 5: HIES Patient: IL-8 + *S. aureus* heatkilled



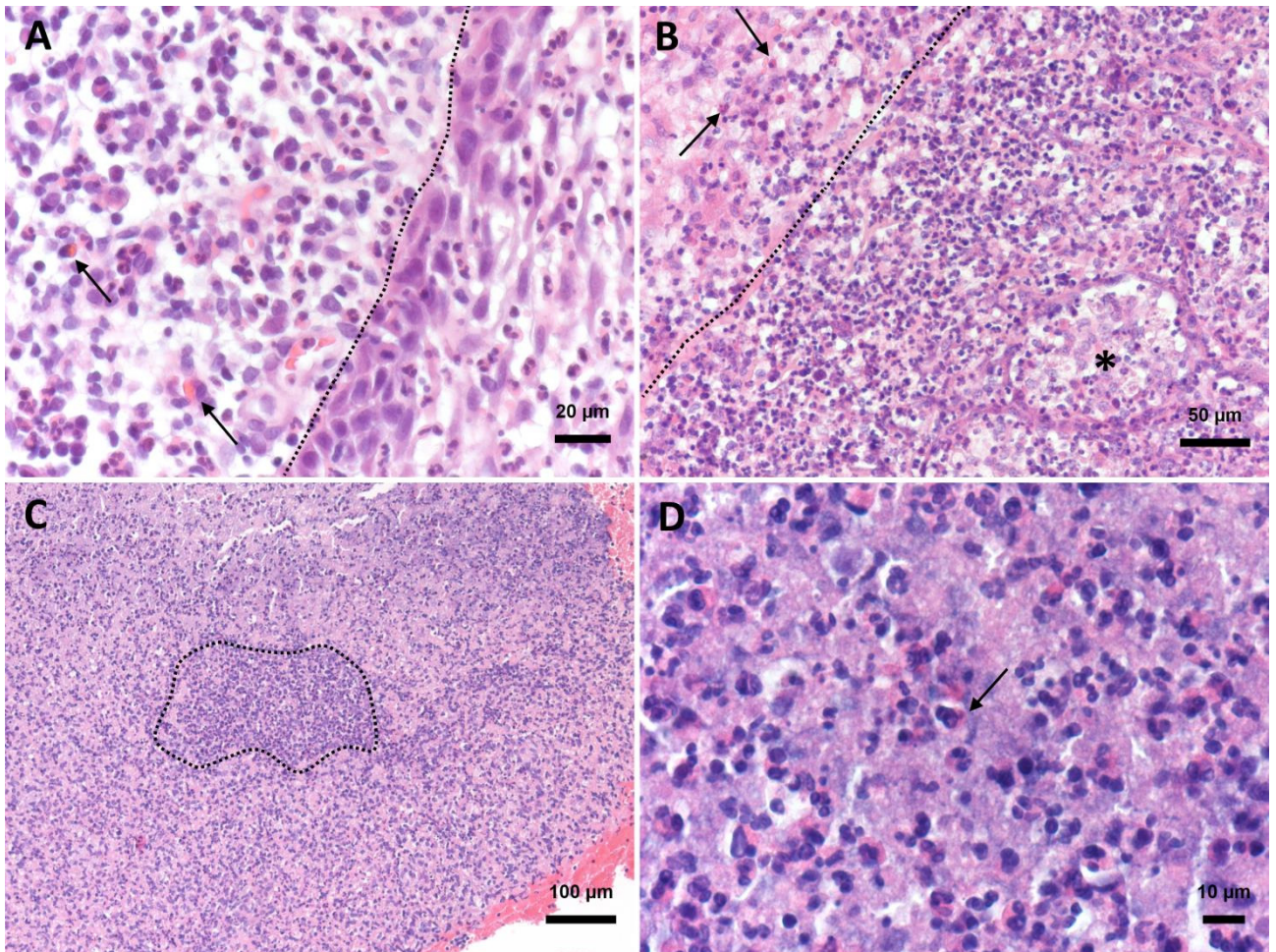
Movie 6: Control: IL-8 + *S. aureus* heatkilled



Movies 1-6. Granulocyte live cell imaging showing accelerated cell death in HIES patients compared to control samples

Granulocytes of HIES patients or controls were stained with cell permeable, DNA-staining dye Hoechst (Life technologies) for 10 min at 37°C, followed by two washes. Cells were then seeded at a concentration of 50.000 cells/well in one 8 well slide (ibidi gmbH) and subsequently stimulated with different agents. Imaging was performed using widefield technique on a Zeiss LSM 710 microscope with a 63x objective in a 37°C, 5% CO₂ incubation chamber over a time period of 14 hours using Zen software. Movies were assembled from the images using Zen software. **Movies 1+2** display NET formation in cells from a HIES patient and the simultaneous control when stimulated with 25 nM PMA. **Movies 3+4** visualise cell death in cells from the HIES patient in comparison to the control when stimulated with vehicle. **Movies 5+6** display cells from the HIES patient and the concomitant control when stimulated with IL-8 (100 ng/ml) and heat-killed *S. aureus*. In order to separate *S. aureus* from cell particles, the bacteria had been pre-stained with both Hoechst (blue) and wheat germ agglutination dye (WGA) 633 (red).

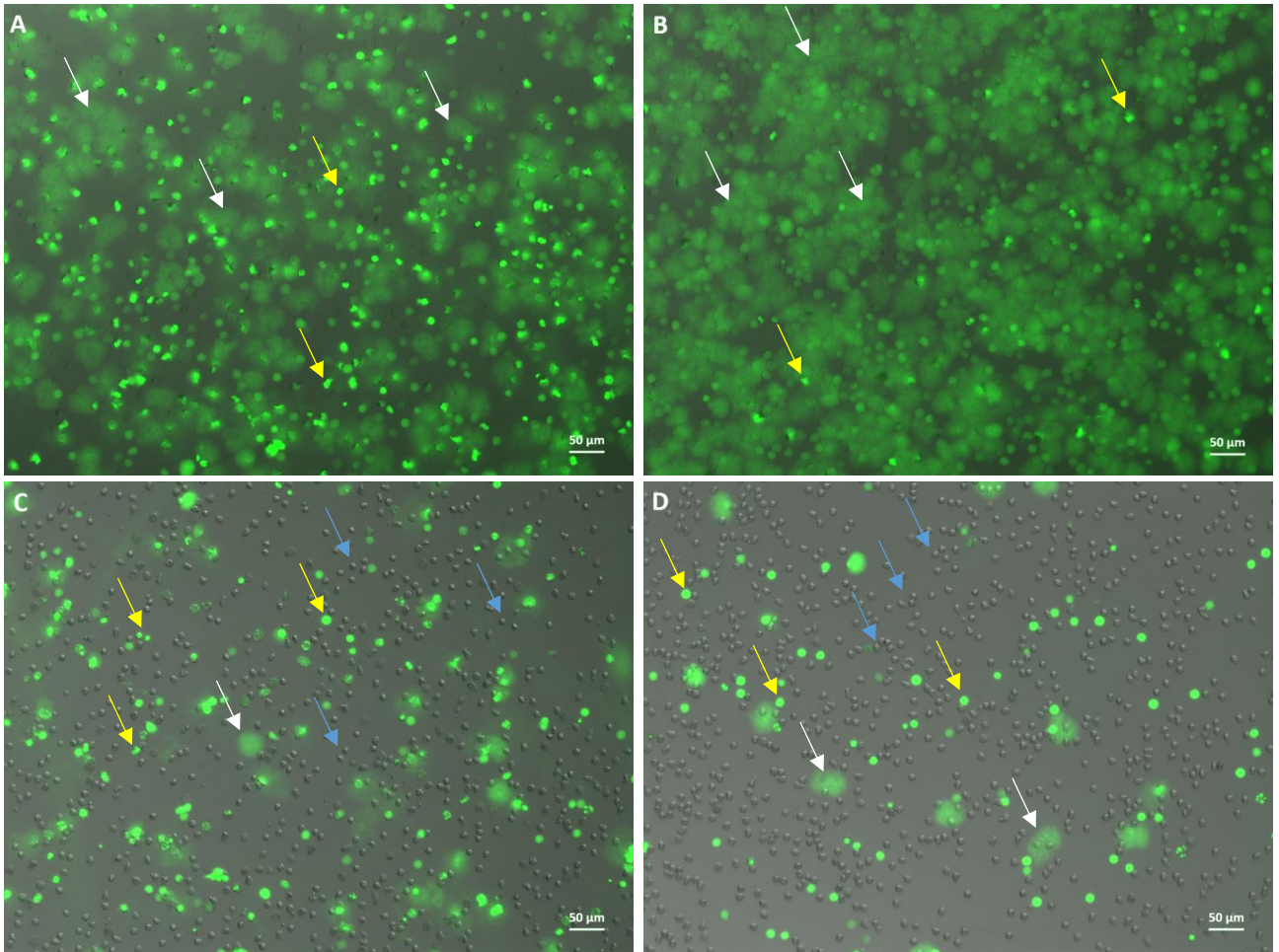
Figures – Supplement



Supplement Figure 1. Presence of neutrophils and eosinophils in histological samples from STAT3-deficient HIES patients.

Displayed are diagnostic histology samples from inflammation sites of STAT3-deficient HIES patients obtained during acute infections (haematoxylin & eosin (H&E) stain, diagnostic routine by the clinical pathology department) (A) Histological sample of a dental abscess showing gingivitis (squamous epithelium basement membrane highlighted by dotted line) with multiple neutrophils and few eosinophils (arrows). (B) Bronchopneumonia with lung abscess. The image shows purulent neutrophilic infiltrates in the central parts (right side of dotted line), surrounded by fibroblasts and inflammatory cells with some eosinophils (left side of dotted line, exemplary eosinophils marked by arrows). Airspaces (asterisk) are filled with macrophages and neutrophil granulocytes. (C) Cervical lymph node abscess showing a large and immature lymphohistiocytic granuloma with suppurative reaction in the centre (highlighted by dotted line). (D) Higher magnification of the suppurative centre in C, displaying debris, neutrophils and marked focal eosinophilia (exemplary eosinophil marked by arrow). Magnifications are indicated by bar and correspond to 40x, 20x, 10x and 60x original magnification for (A-D), respectively.

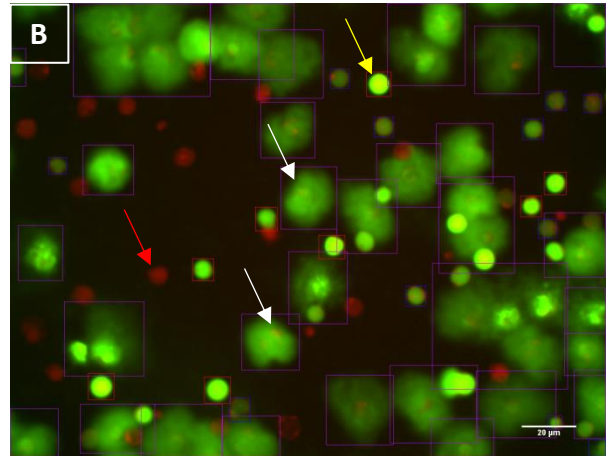
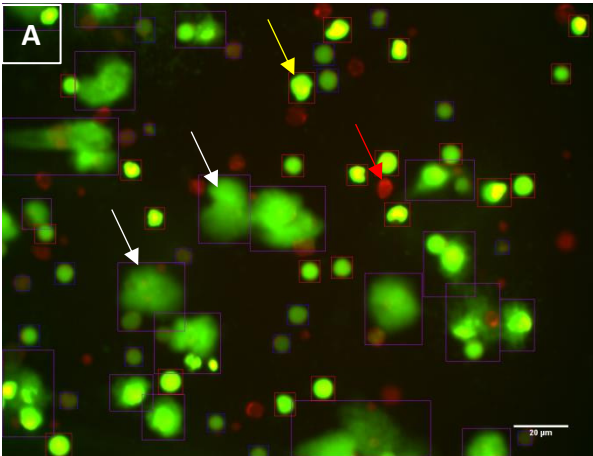
Figures – Supplement



Supplement Figure 2. Microscopic verification of NET formation

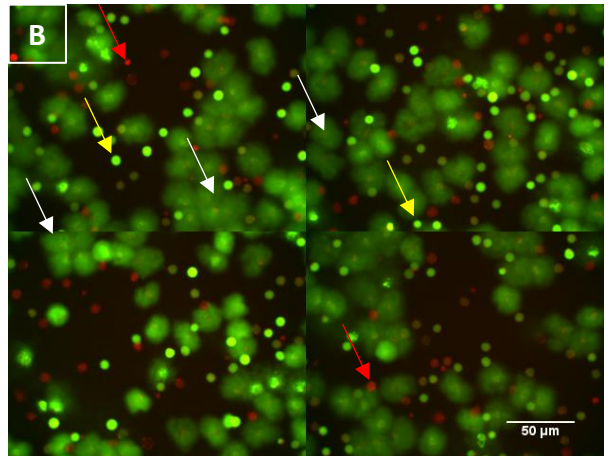
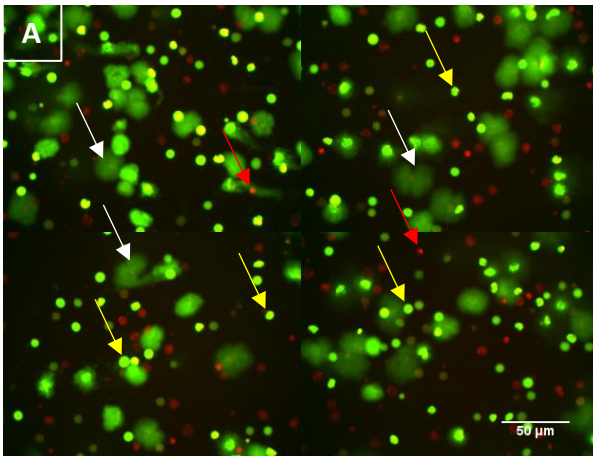
Granulocytes from patients and controls were isolated and stimulated for 2.5 hours with 25 nM PMA or vehicle. Then cells were stained with the cell impermeable DNA-dye Sytox green. The figure shows microscopic images verifying neutrophil extracellular trap (NET) formation in (A) a HIES patient and (B) a concomitant control after stimulation with 25 nM PMA for 2.5 hours. Cloud-like green structures that extend beyond the cell borders are NETs (exemplary white arrows). Necrotic events are small and intensively green (exemplary yellow arrows). The images below show granulocytes from (C) the same HIES patient and (D) the concomitant control after stimulation with vehicle for 2.5 hours. Here, size and amount of intact cells are well appreciable (exemplary blue arrows). Necrotic events are intensively green and small (exemplary yellow arrows) and NETs appear cloud-like with low intensity (exemplary white arrows). All images are taken with 10x magnification using a fluorescence microscope.

Figures – Supplement



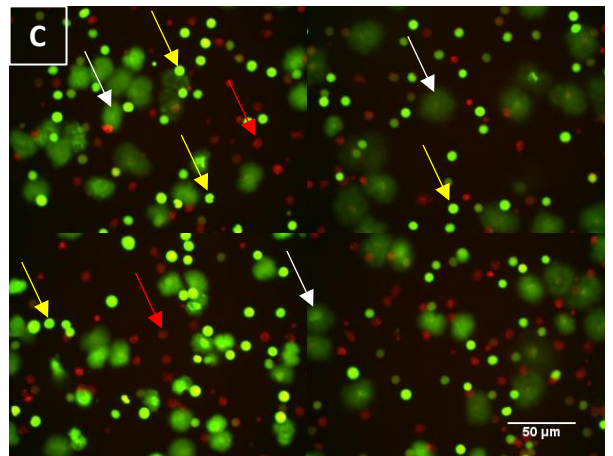
Supplement Figure 3. Assessment of NET formation in HIES patients and controls by automated microscopy

Granulocytes isolated from patients and concomitant controls were stained with CellTracker™ dye prior to stimulation. After two hour incubation with 25 nM PMA or vehicle, the cell impermeable DNA-dye Sytox green was added and the plate was analysed with an automated fluorescence microscope. The figure shows the microscopic assessment of neutrophil extracellular trap formation in (A) a HIES patient and (B) a concomitant control after stimulation with 25 nM PMA. Images are taken with 40x magnification, the scale bar indicates 20 μM. NETs: cloudlike green structures (for examples see white arrows). Necrotic events: small and intensively green (for examples see yellow arrows). Living cells: red (for examples see red arrows).

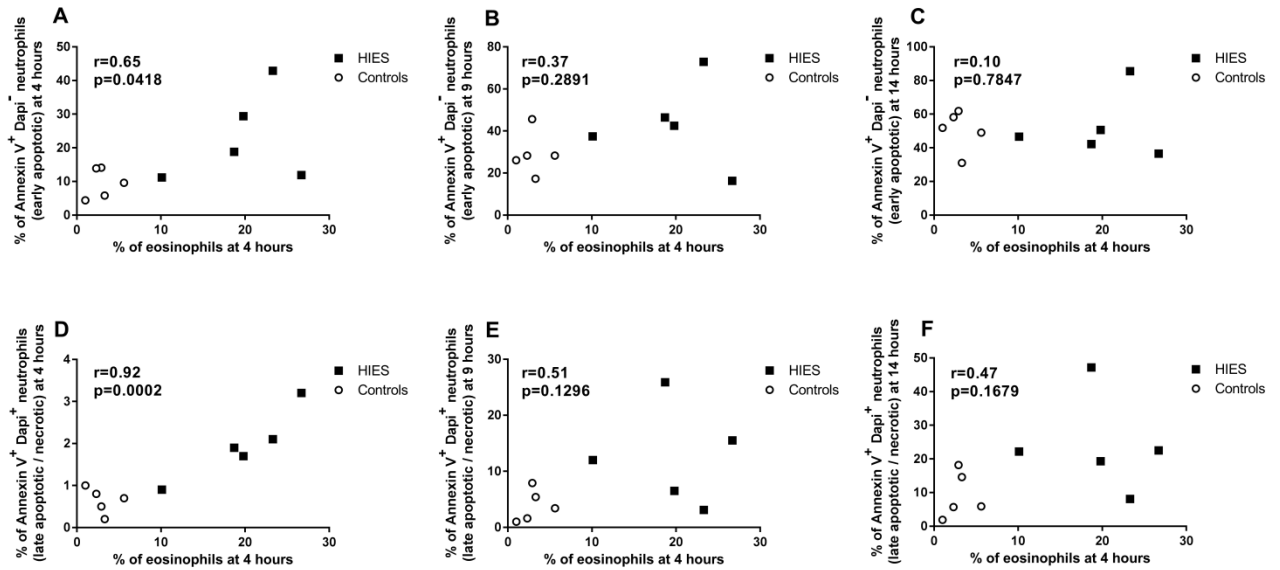


Supplement Figure 4. Effect of prolonged storage at 37°C on cell viability

The figure shows the microscopic assessment of neutrophil extracellular trap formation after stimulation with 25 nM PMA in (A) a HIES patient and (B, C) 2 controls by automatic scanning microscopy (40x magnification, displayed are 4 assembled images per panel, the scale bar indicates 50 μm). NETs: cloudlike green structures (for examples see white arrows). Necrotic events: small and intensively green (for examples see yellow arrows). Living cells: red (for examples see red arrows). The experimental procedure was as described in the legend of Supplement Figure 3. While control B was drawn concomitantly with the patient sample A, control C had been drawn 3 hours earlier and whole blood was stored at 37°C until isolation.



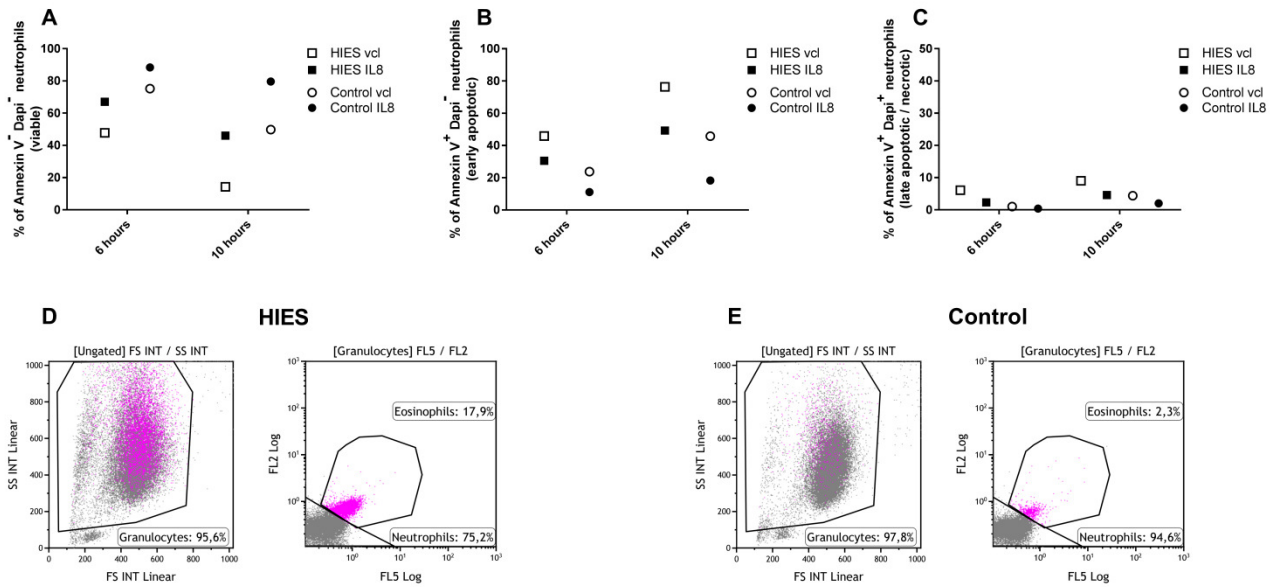
Figures – Supplement



Supplement Figure 5. Correlation between the percentage of eosinophils and the percentage of early apoptotic or late apoptotic/necrotic neutrophils at different time points

Correlation of the percentage of eosinophils among granulocytes at four hours (i.e. at initial measurement) and the percentage of (A-C) early apoptotic (Annexin V⁺ Dapi⁺) or of (D-F) late apoptotic/necrotic (Annexin V⁺ Dapi⁺) neutrophils at four (A,D), nine (B, E) and fourteen (C, F) hours after stimulation with vehicle. Statistical analysis was performed with GraphPad Prism 7 using Pearson correlation. Results from the statistical analysis are displayed within graphs (r =correlation coefficient, $p \leq 0.05$ =significant, $p > 0.05$ =not significant). Displayed are results from five HIES patients and five concomitant controls obtained in four experiments.

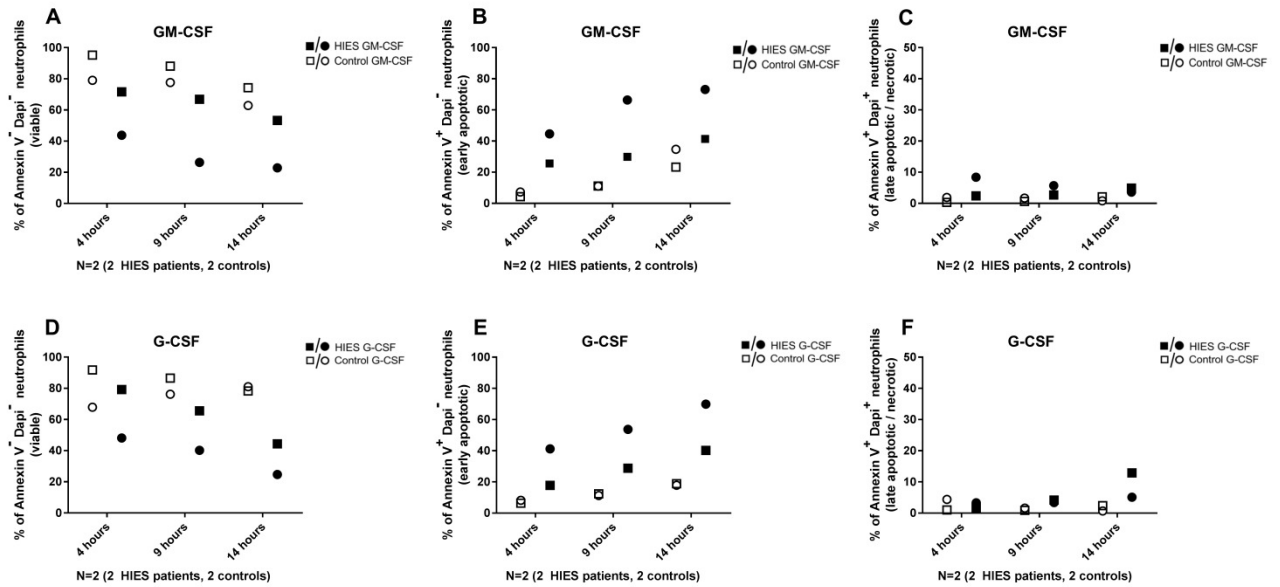
Figures – Supplement



Supplement Figure 6. Neutrophil cell death kinetics following stimulation with IL-8 in one HIES patient

Granulocytes were isolated from the patient and the concomitant control sample followed by IL-8 or vehicle stimulation. At designated time points (six and ten hours), equal amounts of the samples were stained with Annexin V and Dapi and analysed by flow cytometry. Displayed are (A) viable neutrophils (Annexin V⁻ Dapi⁻), (B) early apoptotic (Annexin V⁺ Dapi⁻) neutrophils and (C) late apoptotic/necrotic (Annexin V⁺ Dapi⁺) neutrophils six and ten hours after stimulation with vehicle and IL-8 in a HIES patient and a concomitant control. Data from this experiment could not be pooled with the other experiments shown in Figure 1 due to employment of different time points for the determination of cell death. As FL4 was not activated in the flow cytometer, gating for autofluorescent eosinophils was done at FL2 (Ex 488nm/Em filter 575/26) versus FL5 (Ex 488/Em filter 755LP) for this experiment (D, E).

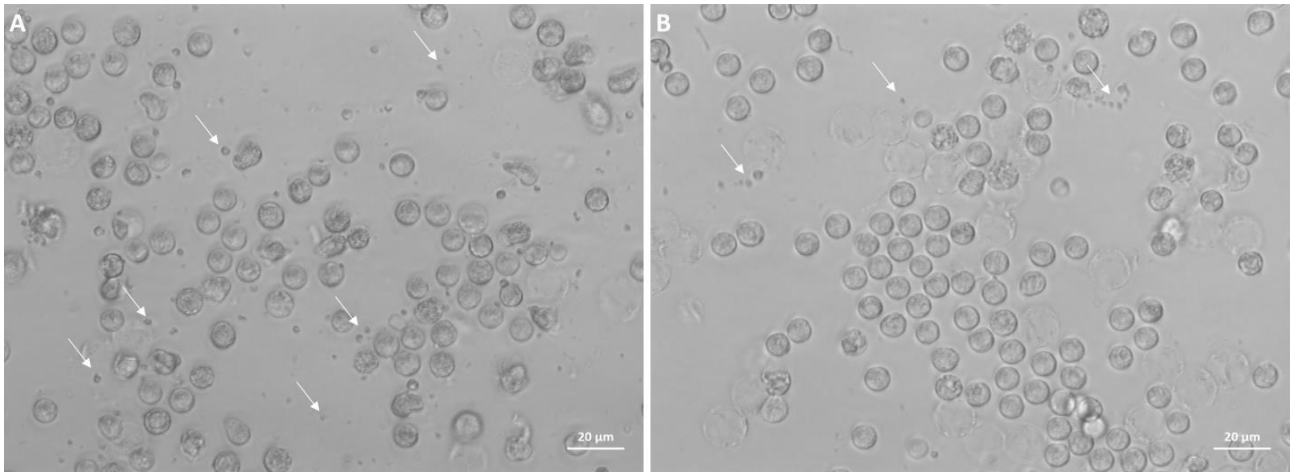
Figures – Supplement



Supplement Figure 7. Neutrophil cell death kinetics following stimulation with GM-CSF and G-CSF in two HIES patients

Granulocytes were isolated from patients and concomitant control samples followed by GM-CSF, G-CSF or vehicle stimulation. At designated time points (four, nine and fourteen hours), equal amounts of the samples were stained with Annexin V and Dapi and analysed by flow cytometry. The figure displays the percentage of (A, D) viable (Annexin V⁻ Dapi⁻), (B, E) early apoptotic (Annexin V⁺ Dapi⁻) and (C, F) late apoptotic/ necrotic (Annexin V⁺ Dapi⁺) neutrophils at four, nine and fourteen hours after stimulation with (A-C) GM-CSF and (D-F) G-CSF in two HIES patients and two corresponding controls. The figure shows data from two individual experiments. Each HIES patient and the respective concomitant control are displayed with the same symbol (dark symbols=HIES patients, light symbols=controls). No statistical analysis was performed since less than three groups are available.

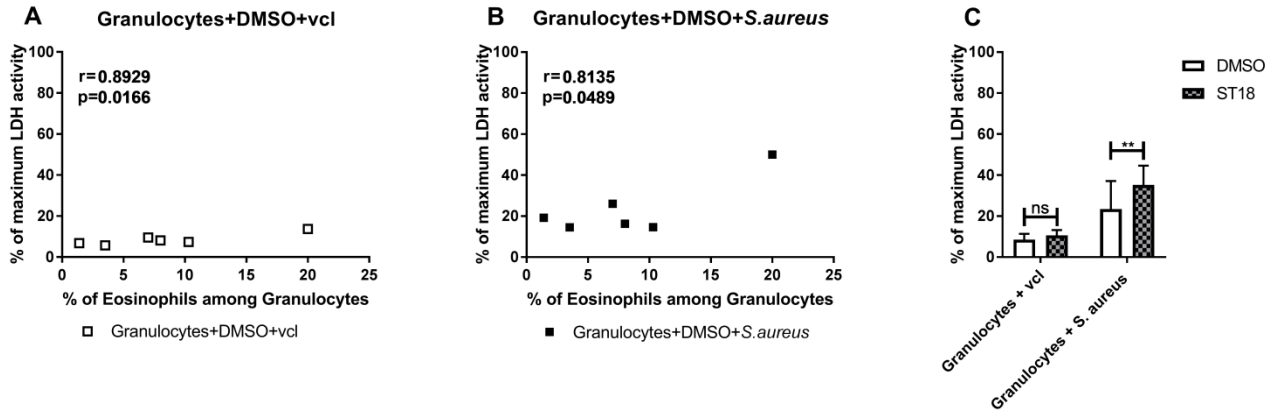
Figures – Supplement



Supplement Figure 8. Enhanced cell destruction visible in granulocyte suspensions from STAT3-deficient HIES patients

Granulocytes were isolated from patients and concomitant control samples. The figure displays a light microscopy image (40x) of unstained, unstimulated granulocyte suspensions six hours after isolation. The samples are derived from (A) a HIES patient and (B) a concomitant control. Examples of cell particles are highlighted by white arrows.

Figures – Supplement



Supplement Figure 9. Correlation between the percentage of eosinophils in granulocyte preparations and the maximum LDH activity

Displayed are results from five individual experiments with cells from six healthy donors with supposedly allergic or asthmatic predisposition where the percentage of eosinophils in the granulocyte preparations and data on LDH-release in granulocytes stimulated with *S. aureus* were available. The same controls were also part of Figure 3A in the manuscript. The granulocyte suspensions were incubated for 60 min with 20 μ M ST18 (STAT3 Inhibitor XVIII, BP-1-102) or DMSO. Subsequently, cells were washed and co-incubated with vcl (vehicle=medium) or *S. aureus* 8325-4 (MOI 5) for 5 hours. Then, LDH release was measured in the supernatants. **(A+B)** Shows the correlation graphs between the percentage of eosinophils in granulocyte suspensions after isolation and the maximum LDH activity for DMSO-treated granulocytes stimulated with **(A)** vcl or **(B)** *S. aureus*. Statistical analysis was performed with GraphPad Prism 7 using Pearson correlation. Results from the statistical analysis are displayed within graphs (r =correlation coefficient, $p \leq 0.05$ =significant, $p > 0.05$ =not significant). Results for ST18-treated samples are not plotted since no significant correlation was found ($r=0.4766$, $p=0.3392$ for granulocytes+ST18+vcl and $r=0.6442$, $p=0.1674$ for granulocytes+ST18+*S.aureus*). **(C)** Displays the corresponding LDH-release in the six samples after stimulation of STAT3-inhibited (ST18) and control (DMSO) granulocytes with vehicle (vcl) or *S. aureus*. Displayed is the mean with SD. RM 2-way Anova with Bonferroni-correction was used for statistical analysis ($p > 0.05$ = not significant (ns); $p < 0.01$ =**).

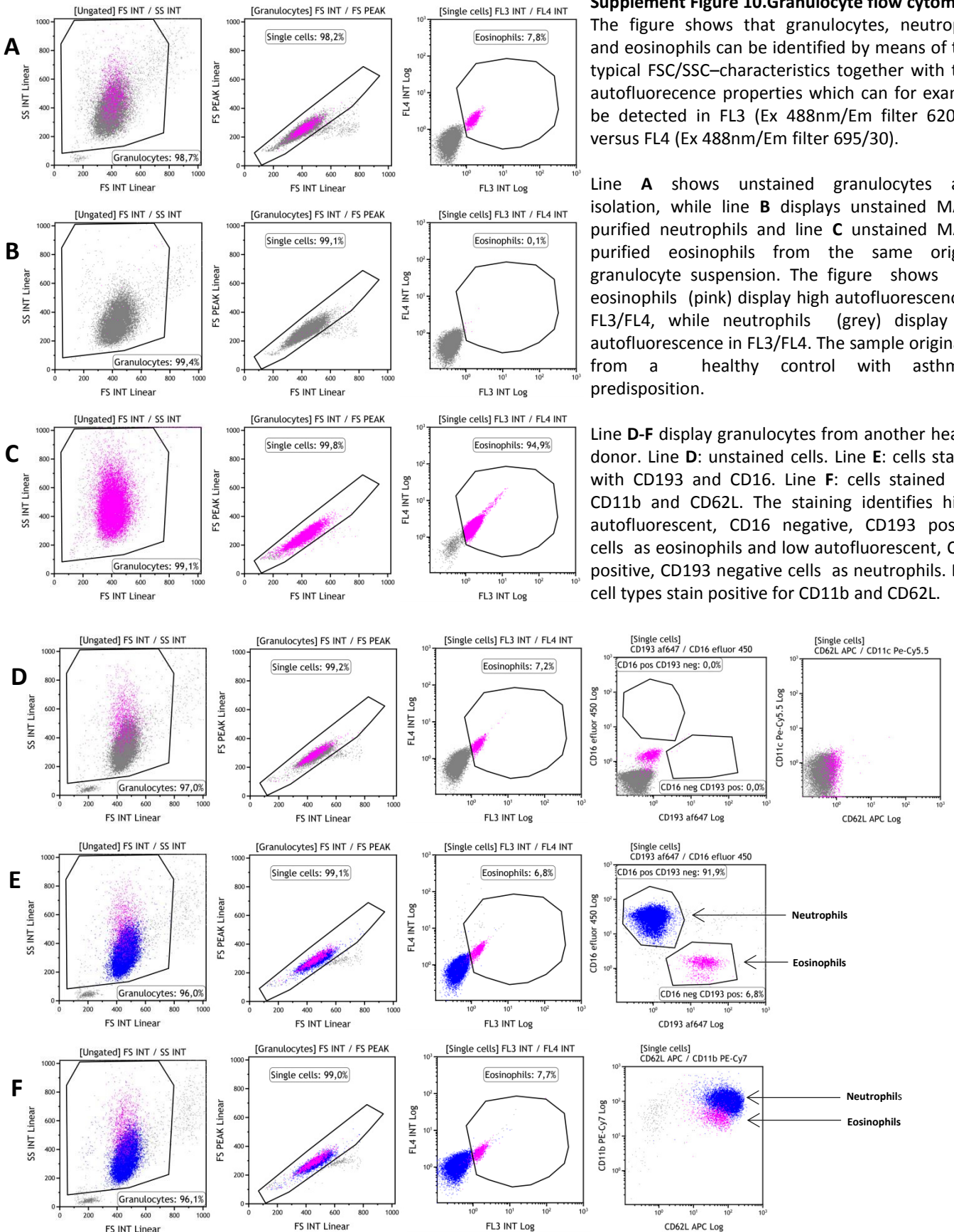
Figures – Supplement

Supplement Figure 10. Granulocyte flow cytometry

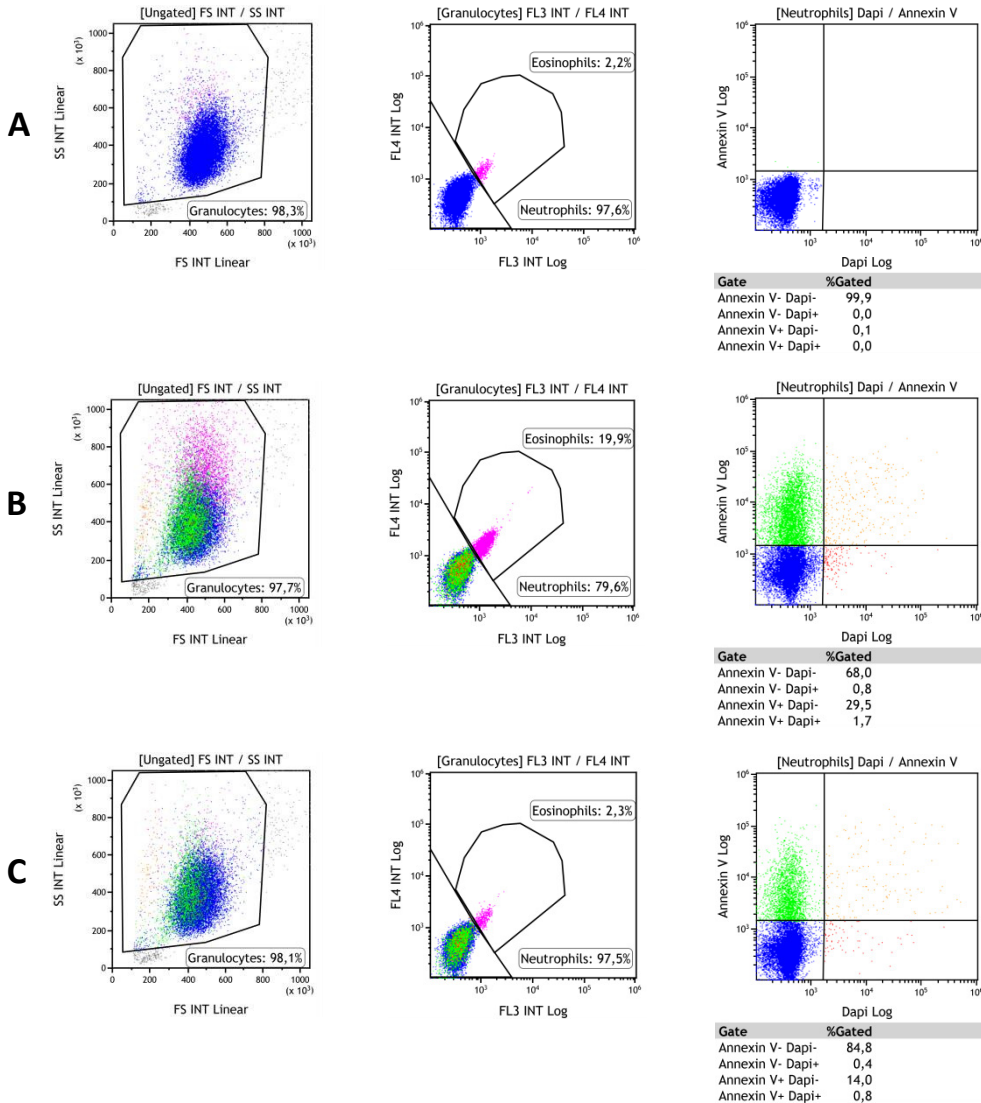
The figure shows that granulocytes, neutrophils and eosinophils can be identified by means of their typical FSC/SSC-characteristics together with their autofluorescence properties which can for example be detected in FL3 (Ex 488nm/Em filter 620/30) versus FL4 (Ex 488nm/Em filter 695/30).

Line **A** shows unstained granulocytes after isolation, while line **B** displays unstained MACS-purified neutrophils and line **C** unstained MACS-purified eosinophils from the same original granulocyte suspension. The figure shows that eosinophils (pink) display high autofluorescence in FL3/FL4, while neutrophils (grey) display low autofluorescence in FL3/FL4. The sample originated from a healthy control with asthmatic predisposition.

Line **D-F** display granulocytes from another healthy donor. Line **D**: unstained cells. Line **E**: cells stained with CD193 and CD16. Line **F**: cells stained with CD11b and CD62L. The staining identifies highly autofluorescent, CD16 negative, CD193 positive cells as eosinophils and low autofluorescent, CD16 positive, CD193 negative cells as neutrophils. Both cell types stain positive for CD11b and CD62L.



Figures – Supplement



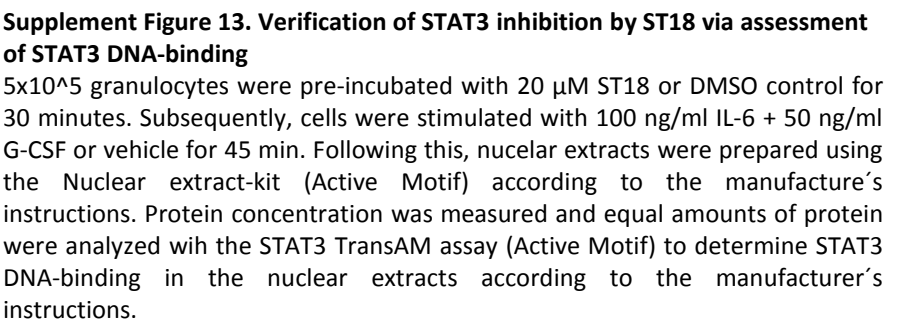
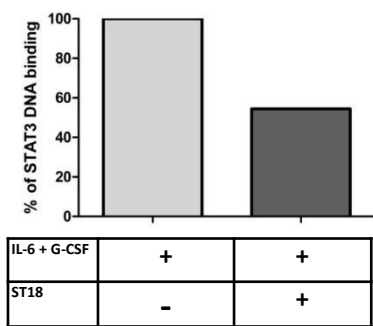
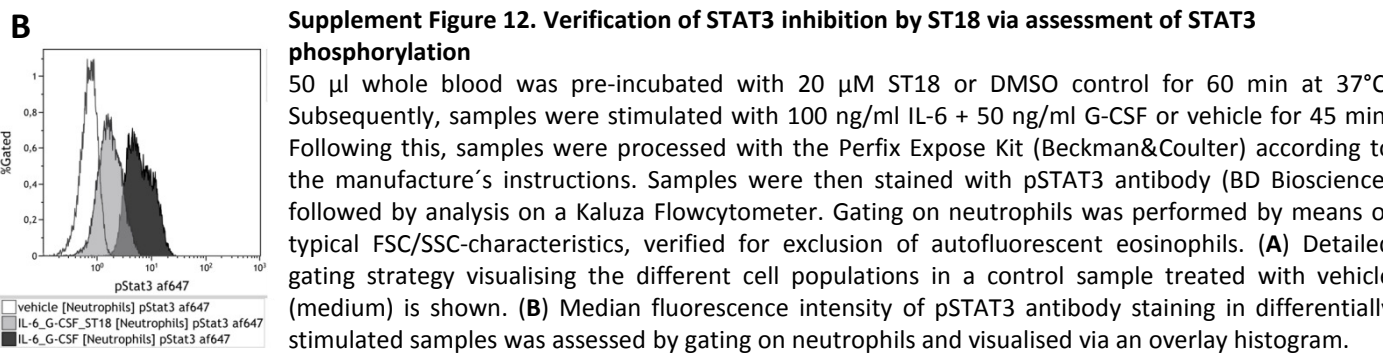
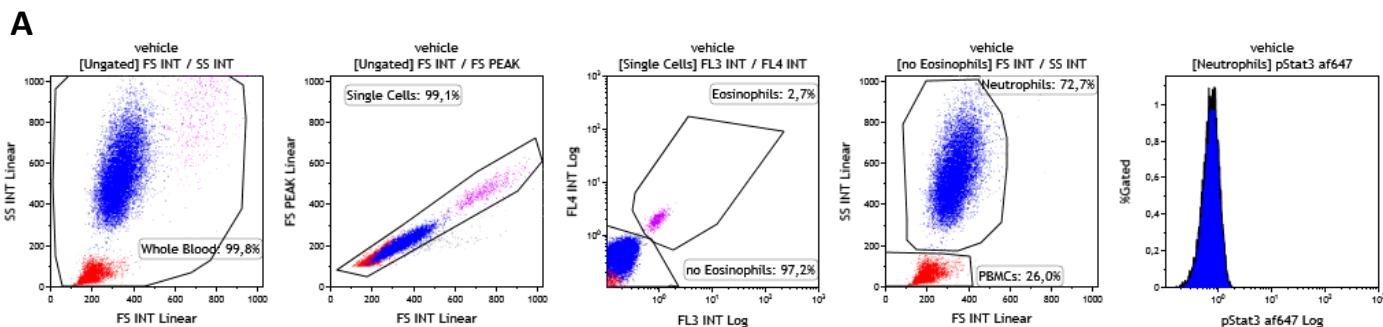
Supplement Figure 11. Gating strategy for cell death analysis of neutrophils by flow cytometry

Within the granulocyte fraction as characterized by the typical FSC/SSC characteristics, neutrophils and eosinophils were generally identified by means of their different autofluorescent properties (for details and gate verification see Supplement Figure 10). Within the neutrophil gate the following categories were used to identify viable, apoptotic and necrotic neutrophils according to the respective Annexin V/Dapi staining:

- Percentage of **viable** neutrophils (Annexin V⁻ Dapi⁻)
- Percentage of **early apoptotic** neutrophils (Annexin V⁺ Dapi⁻)
- Percentage of **late apoptotic/necrotic** neutrophils (Annexin V⁺ Dapi⁺)
- Percentage of **primary necrotic** neutrophils (Annexin V⁻ Dapi⁺)

Images in line **A** are derived from an unstained control sample, while line **B** shows the staining of granulocytes from a HIES patient and line **C** displays the staining of the concomitant healthy control. Dapi staining was always detected in FL9 (Ex 405nm/Em filter 450/50). Depending on whether Annexin V FITC or Annexin V APC was used, Annexin V staining was detected in either FL1 (Ex 488nm/Em filter 525/50) or FL6 (Ex 633nm/Em filter 660BP), respectively. Concomitant patient and control samples were always analysed simultaneously employing identical staining.

Figures – Supplement



Eosinophilia and reduced STAT3 signalling affect neutrophil cell death in autosomal-dominant Hyper-IgE syndrome

Susan Farmand, Bernhard Kremer, Monika Häffner, Katrin Pütsep, Peter Bergman, Mikael Sundin, Henrike Ritterbusch, Maximilian Seidl, Marie Follo, Philipp Henneke, Birgitta Henriques-Normark

Correspondence: Dr. Susan Farmand, Microbial Pathogenesis, M1, Gustav V, Karolinska University Hospital Solna, 17176 Stockholm, Sweden

Review Timeline:	Submission date:	19-Apr-2018
	First Editorial decision:	16-May-2018
	Revision/s received:	29-Aug-2018
	Accepted:	10-Oct-2018

Handling Executive Committee member: Prof. Francesco Annunziato

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision - 16-May-2018

Dear Dr. Farmand,

Manuscript ID eji.201847650 entitled "Eosinophilia and reduced STAT3 Signalling affect Neutrophil Cell Death in autosomal-dominant Hyper-IgE Syndrome" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

Both of our referees raised substantial concerns. You should use the comments of our referees as a guideline to revise the manuscript, tone down the conclusions made and provide the requested controls. However, please note that there is no guarantee of acceptance even if you address all referees concerns. A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees'™ concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Please show the percent of cells within a gate and fluorochrome axis labels in all flow cytometry plots. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

On behalf of Prof. Francesco Annunziato

Dr. Nadja Bakocevic
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

Reviewer: 1

Comments to the Author

The paper by Farmand and colleagues claims that the half-life of neutrophils from autosomal-dominant hyper-IgE syndrome (HIES) is reduced ex vivo. Authors also claim that STAT3-deficient neutrophils are subjected to an increased *S. aureus*-induced cell lysis, dependent on the staphylococcal virulence controlling *agr*-locus.

It is opinion of this Reviewer that the conclusions are too overemphasized. In fact, if one carefully analyzes the results shown in Figure 1, neutrophils from only 2, of the 8, patients display accelerated cell death kinetics.

Also the results shown in figure 2 are, in the opinion of this Reviewer, forced. Data from control donors, in these experiments, are very variable, as also outlined by the authors (Inter-individual differences were observed in both patients and controls). Consequently, the „presumed“, „pag.7: consistent increased in spontaneous and *S. aureus*-induced lytic cell death“ of both granulocyte preparations and highly purified neutrophils from STAT3-deficient patients“ becomes such if compared to the concomitantly analyzed control cells (as shown in each figure panel), but not if compared to other controls (shown in the same figure).

Reviewer: 2

Comments to the Author

This manuscript tackles un-answered questions in the STAT3 deficient HIES patients of why they are susceptible to *Staph aureus* infections and why neutrophil studies have had disparate results. The authors postulate that the lack of Th17 cells do not explain the *Staph* susceptibility and I agree with this suggestion that there are other causes. Compared to other studies of pathogenesis in this disease, the authors postulate a role of the eosinophils for both giving confounding results in prior studies as well as potentially making the patients more prone to *S.aureus* infections. Also decreased STAT3 signaling in itself is contributing to increased neutrophil death from *S. aureus*. Intrinsic neutrophils studies were normal- which is expected as the patients do not have systemic *Staph* infections such as liver abscesses as do the patients with CGD who have truly abnormal neutrophil function. Overall i felt the experiments were well performed and made sense.

few comments:

- 1) I think it would be helpful in understanding the theories of the role of the increased eosinophils to include disease controls of patients with increased eosinophils but without STAT3 mutations. For instance, patents with hypereosinophilic syndromes do not have increase infections- and the difference from the data provided is likely the combination of the high eosinophils plus the abnormal STAT3 signaling as was shown when the STAT3 inhibitor was used. But having other patients with high eosinophils and without the infectious phenotype may be interesting to study.
- 2) Not all patients in this study or in general with STAT3 deficient HIES have high eosinophils but all basically have similar infection phenotypes- any explanation? any correlation with the degree of eosinophilia?
- 3) Compared to CGD, these patients infections are more mucosal and epithelial and the organ infections (like spleen) and even osteomyelitis are more rare- suggesting that there is contribution of both reduced *Staph* killing but potentially something more to the Th17/IL-17 story- would try and tie in the location of the infections to the story.

First Revision – authors’ response - 29-Aug-2018

Dear Professor Annunziato,

Thank you very much for the opportunity to submit a revised, and we believe much improved version of our manuscript entitled “Eosinophilia and reduced STAT3 signalling affect Neutrophil Cell Death in autosomal-dominant Hyper-IgE Syndrome”. We are grateful for the valuable comments of the reviewers. Please find below a point-by-point response to the questions raised. We hope that you find our manuscript suitable for publication in the European Journal of Immunology.

Specific comments to Reviewer 1

1. “The paper by Farmand and colleagues claims that the half-life of neutrophils from autosomal-dominant hyper-IgE syndrome (HIES) is reduced ex vivo. Authors also claim that STAT3-deficient neutrophils are subjected to an increased S. aureus-induced cell lysis, dependent on the staphylococcal virulence controlling agr-locus. It is opinion of this Reviewer that the conclusions are too overemphasized”.

We fully agree with the reviewer that caution is essential in interpreting data from a limited number of patients. While our cohort is small, we would like to emphasize that, to our best knowledge, our study comprises the largest cohort of STAT3-deficient HIES patients in which functional and cell death analysis in neutrophils have been performed. STAT3-deficient hyper-IgE syndrome is a very rare disease with an estimated annual incidence of 1:1 000 000 (www.orpha.net: ORPHA:2314). In Sweden, it is estimated that 10 people are affected (<http://www.socialstyrelsen.se/ovanligadiagnoser/hyper-ige-syndromet>). We are convinced that a small sample size in a rare disease should not discourage the formulation of hypotheses based on the analysis of primary material. Of course, we understand that conclusions based on such a small patient cohort need to be drawn with care. To meet the reviewer’s concern we have inserted a new paragraph in the manuscript on the limitations of the study (**page 14: line 332-341**) and toned down our conclusions (**page 2: line 61-63; page 11+12: deletion of line 277-280; page 13: insertion line 306; page 13: deletion of line 308-310; page 15: line 351**).

2. “In fact, if one carefully analyzes the results shown in Figure 1, neutrophils from only 2, of the 8, patients display accelerated cell death kinetics.”

We disagree with the reviewer that only 2 of the 8 patients display accelerated cell death kinetics. Accelerated cell death - as evidenced by the reduction of viable cells over time - was in fact observed for all of the displayed matched patient-control pairs (**revised Figure 1D**). Functional neutrophil diagnostics requires simultaneous analysis of samples. In particular, when assessing neutrophil cell death kinetics a comparison with the concomitant control is crucial since pre-laboratory variations (e.g. time between sampling, transport of samples, different amounts of available blood) but even minor intra-experimental variations may affect the cells [1-3]. Concomitant analysis of equally treated control samples is thus a standard for functional neutrophil analysis, even in the field of clinical neutrophil diagnostics. We have inserted a sentence in the method part, where we refer to the concomitant sample preparation (**page**

15+16: line 373-374). In order to improve the clarity of our results, we now show each matched pair (HIES-sample and the control-sample that was analysed at the same time) with the same symbol (**revised Figure 1, revised Supplement Figure 7**). Thus, the individual courses are more transparent and the comparison between patients and controls is better comprehensible. The observed differences between patient and controls were statistically significant, despite the variability in the patient and the control groups. The statistical analysis used (repeated-measures (RM) two-way ANOVA with Bonferroni correction for multiple testing) takes into account that samples are paired.

Furthermore, we would like to emphasize that the accelerated cell death in cells from HIES-patients was also observed with other less quantitative methods such as life-cell imaging and microscopy (**Supplement Movies 3-6, Supplement Figure 2C+D and Supplement Figure 8**). Thus, while we agree that the courses are individualistic, accelerated neutrophil cell death was a consistent feature in HIES patients in our study.

“Also the results shown in figure 2 are, in the opinion of this Reviewer, forced. Data from control donors, in these experiments, are very variable, as also outlined by the authors (Inter-individual differences were observed in both patients and controls). Consequently, as stated on page 7, “the consistent increase in spontaneous and S. aureus-induced lytic cell death of both granulocyte preparations and highly purified neutrophils from STAT3-deficient patients” becomes such if compared to the concomitantly analyzed control cells (as shown in each figure panel), but not if compared to other controls (shown in the same figure).”

We agree with this reviewer that analysis of neutrophil death is hampered by high inter-individual variability. Such variability can be expected in cells with a short half live, in particular if sample transport is necessary [1-3]. Thus, we and many other immunodiagnostic laboratories have substantial experience in controlling for the variability due to conditions of sample handling by using shipment controls (i.e. concomitantly drawn and analysed). We acknowledge that pooling of experimental data has to be performed to allow for more robust analysis. Given the limited sample size this was, however, not possible for all experiments in our study. For ethical considerations, patient sampling for research needs to be incorporated into routine blood draws, especially in children. Thus, there are limitations in sample size and availability within our study which we acknowledge in our revised manuscript (**page 8: line 183-184; page 14: line 332-333**). Accordingly, we show the individual experiments in Figure 2 as descriptive data. Notwithstanding the inter-individual variations, the difference between patient and concomitantly analysed control cells was evident in each individual experiment displayed in Figure 2. Thus, we stand to our statement that “...granulocyte preparations and highly purified neutrophils from STAT3-deficient patients consistently showed increased spontaneous and S. aureus-induced lytic cell death as compared to concomitantly analysed control cells.”

We would like to point out that to overcome the problem of the small sample size and lack of statistical analysis in Figure 2 we employed a STAT3 inhibitor in healthy control cells. This approach allowed us to

accumulate an increased number of STAT3-deficient versus control samples and enabled us to perform statistical analysis as shown in Figure 3. Therefore, Figure 3 does not only add new information regarding the relevance of the *agr*-locus for cell lysis but also complements and supports the data on primary patient material presented in Figure 2. We have now added a sentence in the result section that clarifies this point (**page 8: line 184-187**). Control individuals included in Figure 3A were also able to provide higher blood sampling volumes which enabled increased eosinophil purification for experiments. We believe that this point has not become transparent enough in our original manuscript and we have therefore added a sentence referring to this in the revised manuscript (**page 15: line 369-370**).

Specific comments to Reviewer 2

“This manuscript tackles un-answered questions in the STAT3 deficient HIES patients of why they are susceptible to Staph aureus infections and why neutrophil studies have had disparate results. The authors postulate that the lack of Th17 cells do not explain the Staph susceptibility and I agree with this suggestion that there are other causes. Compared to other studies of pathogenesis in this disease, the authors postulate a role of the eosinophils for both giving confounding results in prior studies as well as potentially making the patients more prone to S.aureus infections. Also decreased STAT3 signaling in itself is contributing to increased neutrophil death from S. aureus. Intrinsic neutrophils studies were normal- which is expected as the patients do not have systemic Staph infections such as liver abscesses as do the patients with CGD who have truly abnormal neutrophil function. Overall i felt the experiments were well performed and made sense. “

We thank the reviewer for this positive statement.

“Few comments”:

1.” I think it would be helpful in understanding the theories of the role of the increased eosinophils to include disease controls of patients with increased eosinophils but without STAT3 mutations. For instance, patents with hypereosinophilic syndromes do not have increase infections- and the difference from the data provided is likely the combination of the high eosinophils plus the abnormal STAT3 signaling as was shown when the STAT3 inhibitor was used. But having other patients with high eosinophils and without the infectious phenotype may be interesting to study.“

We agree that the analysis of other patient groups with high eosinophil numbers and without the infectious phenotype would be very interesting. In order to address the reviewer’s suggestion we evaluated more into detail what is known about eosinophilia, infection association and tissue damage. Although patients with hypereosinophilic syndromes (HES) do not show increased infection susceptibility, it is well-known that local eosinophilia can induce organ damage [4]. However, the risk of organ damage is not precisely predicted by the degree of peripheral blood eosinophilia in eosinophilic disorders [5]. Some patients with so-called *hypereosinophilia of undetermined significance* do not show any signs of end-organ damage [6]. This argues that additional factors, either intrinsic to the eosinophils (i.e. genetic variations, activation status) or by the local environment ultimately determine the tissue destruction induced by eosinophils. The

release of their biological active contents by degranulation or cytolysis appears to be a pivotal process in this context [7-9]. This is partly also reflected in Figure 3A. No significant differences were observed between vehicle-treated neutrophils with or without eosinophil supplementation (Figure 3A). Yet, when *S. aureus* was added, LDH release increased in samples which had been supplemented with eosinophils. Thus, eosinophils only impacted cell death when in contact with *S. aureus*. In order to highlight this, we are now also displaying statistics for **Neutrophils+vcl** versus **Neutrophils+Eosinophils+vcl** for both DMSO-treated and ST18-treated neutrophils (**revised Figure 3A**). Furthermore, we have specified in the results section that the lytic effect is observed upon *S. aureus* infection (**page 8: line 194**) and have added a sentence referring to this in the discussion (**page 11: line 274-275**).

Stratification for the diverse underlying diagnoses in patients with hypereosinophilic syndromes (HES) [7, 10] and exclusion of patients receiving immunomodulatory treatments would require access to a large cohort of well-characterized HES patients. Thus, a systematic investigation of other eosinophilic patient groups would be better addressed in a follow-up study. However, we would like to point out that the cells for the experiments displayed in Figure 3A are actually derived from healthy donors with supposedly elevated eosinophil numbers but without any infection history. Initially, these specific controls were recruited in order to be able to run the experiments with autologous eosinophil supplementation, since isolation of sufficient numbers of eosinophils is limited in non-eosinophilic donors. The inclusion criteria for otherwise healthy individuals (i.e. no infectious history, no acute or chronic illness) donating blood for experiments in Figure 3A was “suspected or proven eosinophilia” related to “predisposition to asthma or allergies” without specific medication. Following the reviewer’s suggestion we went back to the data to determine whether there was a correlation between the percentage of eosinophils in the granulocyte fraction and the LDH release of granulocyte preparations. Documented eosinophil concentrations in the granulocytes preparations (as determined by flow cytometry) and data on granulocytes treated with ST18 and *S. aureus* were available for six of the nine subjects. We have created a new figure to show this data (**Supplement Figure 9**). Not all of these study subjects were in fact eosinophilic at the time of analysis and the documented percentage of eosinophils in the initial granulocyte preparation varied highly between 1.4 and 20% (**Supplement Figure 9A+B**). In this limited amount of samples there was a mild correlation between the percentage of eosinophils among the granulocytes and the LDH release in DMSO-incubated control granulocytes for both unstimulated (i.e. vcl-treated) and *S. aureus* stimulated sample (**Supplement Figure 9A+B**). The correlation was not evident anymore when pre-incubating with ST18 ($r=0.4766$, $p=0.3392$ for granulocytes+ST18+vcl and $r=0.6442$, $p=0.1674$ for granulocytes+ST18+*S.aureus*). The healthy donor with the highest eosinophil concentration of 20%, which was in the range of what we saw in the HIES-patients (**Table 1**), showed markedly increased LDH-release, particularly following *S. aureus* stimulation (**Supplement Figure 9**). We believe that the results displayed in Supplement Figure 9 strengthen the observation that elevated eosinophil concentrations during granulocyte isolation may increase the baseline of spontaneous and pathogen-induced cell death. Yet, even though four of these six

samples were derived from eosinophilic individuals as defined by eosinophil concentration >7%, there was still a strong effect of STAT3 inhibition on LDH release when stimulated with *S. aureus* (**Supplement Figure 9C**). Thus, the effect of STAT3 inhibition on *S. aureus*-induced cell death appears to be independent from the effect of the eosinophils. We are referring to this new figure in the result section (**page 8: line 195-200**) and in the discussion (**page 12: line 287-289**).

2) “*Not all patients in this study or in general with STAT3 deficient HIES have high eosinophils but all basically have similar infection phenotypes- any explanation? any correlation with the degree of eosinophilia?*”

This point is highly interesting and tackles one of the major questions in STAT3-deficient HIES. In our cohort, we did not observe a direct correlation between the degree of blood eosinophilia and the clinical phenotype. It appears that the effect of eosinophils during experimental workup as well as during infections is rather related to the relative proportion of eosinophils among granulocytes than to blood eosinophilia per se. In line with this, relative eosinophil numbers were elevated in the granulocyte preparation in all of our patients even in patient #1 with documented normal absolute blood eosinophil numbers (<500/ μ l) (**Table 1; page 5: line 130-132; page 10: 238-241**). Furthermore, we noticed that eosinophils can be detected in the tissue during acute infection (**Supplement Figure 1; Table 2**). Thus, lack of eosinophilia in the blood does not exclude that eosinophils are disproportionally elevated in granulocyte preparations intended for neutrophil testing or during acute infections.

While the infection susceptibility and even laboratory findings are similar in all patients, the degree of disease severity and residual damages varies (**page 5: line 120-121; page 6: line 135-136**). In our cohort, the paediatric patients appeared much more affected by infections than the two adult patients (HIES 6 and HIES 7). Still, one adult patient also had bronchiectasis but only one pneumonia was documented in his chart (**Table 2**). Although our group is too small to draw any firm conclusion, potential explanations may be a bias induced by early diagnosis or variable clinical follow up [11], or the contribution of other immunological alterations in HIES that are mentioned in our study (**Table 1; page 5: line 123-127**). As our study focuses on neutrophil functions with a particular emphasis on the response to *S. aureus* in HIES, we do, however, not elaborate in more detail on other known immunological alterations without recognized association to staphylococcal infections.

A complicating factor in STAT3 deficiency is that the lack of STAT3 as a central regulator of immune response may affect both pro- and anti-inflammatory signalling pathways. In addition, STAT3 is ubiquitously expressed and thus exerts diverse effects not only on the immune system but most likely also on the epithelium [12]. We are referring to this fact more clearly in our revised manuscript (**page 13: line 311-312**). Furthermore, we address that the small sample size limits the possibility of correlating experimental findings and the phenotype in individual patients (**page 14: line 335-337**).

3)“*Compared to CGD, these patients infections are more mucosal and epithelial and the organ infections (like spleen) and even osteomyelitis are more rare- suggesting that there is contribution of both reduced*

Staph killing but potentially something more to the Th17/IL-17 story- would try and tie in the location of the infections to the story”.

We are grateful for this suggestion and have added a paragraph in the discussion highlighting the relevance of the location of the infection (**page 13: Line 311-317**). Given the ubiquitous expression of STAT3, it is likely that the combination of local factors at the mucocutaneous sites and in immune cells may ultimately shape the outcome. We believe that this interaction may be particularly interesting to address in further studies (**page 14: line 339-341**). We also highlight that the maintained neutrophil functions (e.g. killing, ROS-production, NETs release) help to prevent invasive organ infections (**page 13: line 317-318**). As mentioned by the reviewer, this is in contrast to other diseases with known susceptibility to *S. aureus* and *Candida* and altered neutrophil functions, such as CGD. As our article has already reached the maximum recommended limit of words and this response letter will be available online we do, however, not refer to CGD specifically in the manuscript.

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12 Farmand, S. and Sundin, M., Hyper-IgE syndromes: recent advances in pathogenesis, diagnostics and clinical care. *Curr Opin Hematol*. 2015. **22**: 12-22.

Changes in the Tables

Table are now provided in an editable word format as requested. Some minor orthographic errors were corrected. No changes in data content were made.

List of Changes made in the Supplement

In order to comply with Journal Standards the following changes were made:

Page1, Movies 1-6: A scale was added for each image. Data interpretation in the legend was deleted.

Page 2, Supplement Figure 1: The magnification was mentioned for each image displayed by adding the following sentence in the legend: "Magnifications are indicated by bar and correspond to 40x, 20x, 10x and 60x original magnification for (A-D), respectively."

Page 3, Supplement Figure 2: The figure legend was modified by adding details on the experimental procedure. Data interpretation within the legend was deleted.

Page 4, Supplement Figure 3 and Supplement Figure 4: Magnifications and scales were added for all images. The legends of both figures were modified by adding details on the experimental procedure. Data interpretation within the legends was deleted.

Page 5, Supplement Figure 5: The following sentence was inserted at the end of the legend: "Displayed are results from five HIES patients and five concomitant controls obtained in four experiments."

Page 6, Supplement Figure 6: The displayed flow cytometry plots in **Supplement Figure 6D+E** were supplemented with the axis scale and the percentage of the respective populations within the gates. Excitation/emission values of the employed fluorochrome detectors and details on the experimental procedure were inserted in the legend.

Page 7, Supplement Figure 7: The figure display was changed by using the same symbol for each respective HIES-control pair. This modification in the figure display allows to follow the individual course of each sample and to visually compare each HIES patient to the concomitant control. Details on the experimental procedure were inserted in the legend and the following sentence was added to clarify the figure layout: "Each HIES patient and the respective concomitant control are displayed with the same symbol (dark symbols=HIES patients, light symbols=controls)."

Page 8, Supplement Figure 8: The legend was modified by adding details on the experimental procedure. Data interpretation within the legend was deleted.

Page 9, Supplement Figure 9: A new figure was inserted showing the correlation between the percentages of eosinophils in granulocyte suspensions with maximum LDH activity. The numbering of the subsequent supplement figures was changed accordingly.

Page 10, Supplement Figure 10 (former Supplement Figure 9): The gating strategy to discriminate highly autofluorescent eosinophils from neutrophils is displayed in more detail. Percentages of relevant cell populations and axis scales are displayed. In addition, an unstained sample was inserted in line **D** showing the same plots as used in line **E** and **F**, where cells were stained to identify neutrophils and eosinophils depending on their respective CD16/CD193 (line **E**) and CD11b/CD62L (line **F**) staining patterns. The last part of legend was slightly changed to address this.

Page 11, Supplement Figure 11 (former Supplement Figure 10): The gating strategy was complemented by adding the percentages of relevant cell populations and axis scales to the plots. The legend was re-written with more details including the employed fluorochromes and their respective excitation/emission characteristics.

Page 12, Supplement Figure 12 and 13 (former Supplement Figure 11 and 12): The gating strategy in **Supplement Figure 12** is displayed in more detail. Data interpretation within the legends of both figures was deleted.

Editorial comments

1. "You should use the comments of our referees as a guideline to revise the manuscript, tone down the conclusions made and provide the requested controls."

We have shortened our manuscript but also included new paragraphs in order to meet the reviewers concerns. In particular, we have inserted a paragraph outlining the limitations of the study (edited manuscript, **page 14: line 332-341**) and toned down the conclusions as asked for (**page 2: line 61-63; page 11+12: deletion of line 277-280; page 13: insertion line 306; page 13: deletion of line 308-310; page 15: line 351**).

Controls with elevated eosinophil numbers, but without infections, were already part of the study. This has apparently not become clear enough in our initial version. In our revision we highlight this information (**page 15: line 366-369**). In addition, we have added a new figure which elaborates on the correlation between eosinophilia and LDH-release from granulocyte preparations (**Supplement Figure 9**). Finally, we have provided a point-by point response to address the reviewers concerns below and explained our view on including patients with other diseases.

2. "You should also pay close attention to the editorial comments included below."

We have made several changes, particularly in the supplement, to comply with the suggestions in the editorial comments. For more details please see the **list of changes made in the supplement** at the end of this reply to the referees.

3. "In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments."

We have edited the figure legends in the manuscript and in the supplement to follow Journal standards as outlined in the editorial comments.

4. "Please show the percent of cells within a gate and fluorochrome axis labels in all flow cytometry plots."

We have added the percentage of cells within a gate and fluorochrome axis labels in the flow cytometry plots. The panels in **Figure 1A-C** consist of the same plot. The percentage of cells is shown only within the gate of interest, since we want to highlight the identification of the respective cell population in these images. However, the complete gating strategy for these plots, including the percentage of cells within all gates and the analysis in general, is shown in detail in the supplement (**Supplement Figure 10 and 11**). We have also specified excitation as well as emission filters for all applied fluorochrome detectors. 2

Second Editorial Decision - 24-Sep-2018

Dear Dr. Farmand,

It is a pleasure to provisionally accept your manuscript entitled "Eosinophilia and reduced STAT3 Signalling affect Neutrophil Cell Death in autosomal-dominant Hyper-IgE Syndrome" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: <https://onlinelibrary.wiley.com/toc/15214141/0/ja>). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Laura Soto Vazquez

on behalf of Prof. Francesco Annunziato

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