IL-17/Th17 Pathway Is Activated in Acne Lesions

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Abstract

The mechanisms of inflammation in acne are currently subject of intense investigation. This study focused on the activation of adaptive and innate immunity in clinically early visible inflamed acne lesions and was performed in two independent patient populations. Biopsies were collected from lesional and non-lesional skin of acne patients. Using Affymetrix Genechips, we observed significant elevation of the signature cytokines of the Th17 lineage in acne lesions compared to non-lesional skin. The increased expression of IL-17 was confirmed at the RNA and also protein level with real-time PCR (RT-PCR) and Luminex technology. Cytokines involved in Th17 lineage differentiation (IL-1β, IL-6, TGF-β, IL23p19) were remarkably induced at the RNA level. In addition, proinflammatory cytokines and chemokines (TNF-α, IL-8, CSF2 and CCL20), Th1 markers (IL12p40, CXCR3, T-bet, IFN-γ), T regulatory cell markers (Foxp3, IL-10, TGF-β) and IL-17 related antimicrobial peptides (S100A7, S100A9, lipocalin, hBD2, hBD3, HCAP18) were induced. Importantly, immunohistochemistry revealed significantly increased numbers of IL-17A positive T cells and CD83 dendritic cells in the acne cells. In summary our results demonstrate the presence of IL-17A positive T cells and the activation of Th17-related cytokines in acne lesions, indicating that the Th17 pathway is activated and may play a pivotal role in the disease process, possibly offering new targets of therapy.

Introduction

Acne is a common disease characterized by androgen dependence, follicular hyperkeratosis, increased sebum excretion, colonization with Propionibacterium acnes (P. acnes) and inflammation. The earliest subclinical acne lesion is the microcomedo, which results from increased proliferation and retention of infundibular keratinocytes [1]. The cytokine IL-1β may have a role in the initiation of microcomedos, by its ability to induce hypercornification of keratinocytes [2,3]. The formation of microcomedos is preceded by mononuclear infiltrates consisting mainly of CD4+ T-cells and CD68+ macrophages [3]. CD4+ T-cells are the major leukocytes in the early (6–72 h) inflammatory infiltrates in acne lesions, with a small portion of CD1+ dendritic cells. Neutrophils emerge increasingly numerous in the 24 h and 72 h lesions, which are then clinically classified as pustules. At later time points CD8+ cells infiltrate in the lesions [4,5].

It has been suggested that P. acnes is involved in the triggering of inflammatory acne via Toll-like receptors (TLRs). The importance of TLR-mediated immune response is supported by the presence of TLR2 expressing cells in inflammatory acne lesions. Furthermore, non-immune cells like keratinocytes and sebocytes express functional TLR2 [6–12]. P. acnes is considered to be a trigger of exaggerated TLR2 mediated immune responses in acne [13]. TLR2 receptors are involved in the recognition of wide array of microbial molecules mainly in gram-positive bacteria, and also yeasts [14]. Recently, P. acnes was shown to activate Nod-like receptor 3 (NLRP3) inflammasome in monocytic cells leading to the production of IL-1β [15,16]. However, it is still unclear whether P. acnes can initiate comedogenesis or early phase inflammatory reaction in acne [17]. Also other triggers than P. acnes for the early inflammatory cascades in acne lesion formation for example leukotriens or free fatty acids should be considered [18].

In addition to innate immunity, also adaptive immunity, and especially the Th17 pathway, may contribute significantly to the inflammatory response in acne [18], [19]. Previously P. acnes has
been shown to stimulate the production of IL-17A and IFN-γ in peripheral blood mononuclear cells (PBMCs) [20–22]. Moreover, the increased expression of cytokines and other inflammatory markers such as IL-1α, beta-defensins 1 and 2, TNF-α, IL-1β, IL-8, IL-10, matrix metalloproteinases MMP-1, MMP-3, MMP-9, CXCL-2 was found in acne lesions in vivo [3,23–26].

This study was based on the analyses of skin biopsies from clinically early looking inflamed acne lesions (comedones with minimal erythematos flare or small papules). The study material was recruited by two clinical centers - Oulu, Finland and Berlin, Germany - with independent groups of patients with acne vulgaris, as well as psoriasis patients and healthy volunteers as controls. Our results show that, as in psoriasis, the Th17 pathway is significantly up-regulated both at the RNA and protein level in lesions of acne vulgaris. The results suggest a novel pathomechanism in inflammatory acne, and open up the possibility for a new class of therapeutics targeting the Th17 system in severe acne.

Materials and Methods

Ethics statement

The studies presented in this manuscript have been approved by local Ethics committees of Oulu University Hospital in Finland and the Charite Universitätsmedizin Berlin in Germany. The biopsies were taken with informed and written consent. All clinical investigations were conducted in accordance with the Declaration of Helsinki Principles.

Table 1. Clinical characteristics of acne patient group from Finland.

| Subject | Age | Gender | Acne severity | Technology |
|---------|-----|--------|---------------|------------|
| 1       | 21  | female | 2             |            |
| 2       | 24  | female | 1             |            |
| 3       | 21  | female | 2             |            |
| 4       | 17  | female | 3             |            |
| 5       | 34  | female | 1             |            |
| 6       | 25  | female | 1             |            |
| 7       | 29  | female | 2             |            |
| 8       | 22  | male   | 3             |            |
| 9       | 29  | male   | 3             |            |
| 10      | 21  | male   | 2             |            |
| 11      | 26  | female | 2             |            |
| 12      | 22  | male   | 3             |            |
| 13      | 19  | male   | 2             |            |
| 14      | 27  | male   | 3             |            |
| 15      | 25  | male   | 1             |            |
| 16      | 26  | male   | 2             |            |
| 17      | 25  | male   | 4             |            |
| 18      | 36  | female | 1             |            |
| 19      | 31  | female | 2             |            |
| 20      | 18  | female | 2             |            |

Mean = 24.9 ± 5.1

1Data are expressed as mean ± SD.
2Acne severity at the biopsy site (back or chest) was assessed by Leeds revised acne grading system in Finnish patient group.
3The technology, which was used to investigate the biopsies taken from the patients.

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Subjects and sampling

The study was performed in two clinical centers in Oulu, Finland and Berlin, Germany. A total of 56 acne patients with moderate to severe acne vulgaris were included in the study. Clinical characteristic of patients is presented in Tables 1 and 2. Control subjects comprised patients with psoriasis (n = 9, age range 28–65; mean 52.9, not age-matched) and healthy volunteers that had never had acne (n = 6, age range 23–36; mean 26.5). The inclusion criteria for psoriasis patients were chronic plaque psoriasis, not receiving systemic or UV-treatment for at least 1 month before sampling. 3 mm (German cohort) or 4 mm (Finnish cohort) punch biopsies were obtained under local anesthesia from the back or chest of acne patients from lesional and non-lesional skin. The acne lesions obtained by biopsy were identified clinically as early inflammatory acne lesions meaning comedones which were shading into small red papules (Figure 1). The control skin biopsies were taken from the back of psoriasis patients lesional and non-lesional skin and healthy volunteers back skin. Utilization of all biopsies is described in Figure S1.

Affymetrix

Biopsies for the microarray analysis were stored in RNA Stabilization Reagent. For RNA extraction the samples were homogenized with a potter in Qiagen lysis buffer. Total RNA was extracted using miRNeasy extraction kits according to manufacturer’s protocol. RNA quantity was measured using Nanodrop
spectrophotometer ND8000. RNA quality was monitored using a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Probes were synthesised and then hybridized on Affymetrix U133 Plus 2.0 chips (Affymetrix, Santa Clara, CA). All chips were normalized using RMA method [27]. Only Affymetrix identifiers (IDs) with expression $\geq 2^{\text{exp}6}$ in at least one condition were selected. Finally, 38014 out of 54675 initially present IDs were selected. Data analysis was performed on Array Studio software (OmicSoft, Cary, NC). Mean expression levels were obtained by calculating the geometrical means of the RMA-normalized data for involved and non-involved sample groups, respectively. A two sided paired T test was performed using Array Studio (OmicSoft Corporation, USA), to determine which genes were significantly differentially expressed between involved and non-involved groups, and Benjamini-Hochberg false discovery rate (FDR).

Table 2. Clinical characteristics of acne patient group from Germany.

| Subject | Age | Gender | Acne severity | Technology |
|---------|-----|--------|---------------|------------|
| 1       | 18  | male   | Severe        | Affymetrix and RT-PCR |
| 2       | 19  | male   | Severe        |            |
| 3       | 20  | female | Moderate      |            |
| 4       | 21  | male   | Severe        |            |
| 5       | 28  | male   | Moderate      |            |
| 6       | 26  | male   | Severe        |            |
| 7       | 22  | male   | Severe        |            |
| 8       | 30  | male   | Mild          |            |
| 9       | 23  | male   | Severe        |            |
| 10      | 24  | male   | Moderate      |            |
| 11      | 19  | male   | Severe        |            |
| 12      | 26  | male   | Mild          |            |
| 13      | 25  | male   | Moderate      | Luminex    |
| 14      | 20  | male   | Severe        |            |
| 15      | 28  | male   | Moderate      |            |
| 16      | 27  | male   | Moderate      |            |
| 17      | 21  | male   | Moderate      |            |
| 18      | 22  | male   | Mild          |            |
| 19      | 25  | male   | Moderate      |            |
| 20      | 19  | male   | Severe        |            |
| 21      | 20  | male   | Mild          |            |
| 22      | 29  | female | Moderate      |            |
| 23      | 31  | male   | Severe        |            |
| 24      | 31  | male   | Moderate      |            |
| 25      | 18  | male   | Severe        | Immunohistochemistry |
| 26      | 19  | male   | Moderate      |            |
| 27      | 29  | male   | Severe        |            |
| 28      | 24  | male   | Severe        |            |
| 29      | 30  | male   | Severe        |            |
| 30      | 20  | male   | Moderate      |            |
| 31      | 19  | male   | Moderate      |            |
| 32      | 19  | female | Moderate      |            |
| 33      | 27  | female | Severe        |            |
| 34      | 29  | male   | Moderate      |            |
| 35      | 22  | male   | Severe        |            |
| 36      | 21  | male   | Moderate      |            |

Mean $23.6 \pm 4.2$

1. Data are expressed as mean ± SD.
2. Acne severity at the biopsy site (back) was assessed by Global Evaluation Acne scale in German patient group.
3. The technology, which was used to investigate the biopsies taken from the patients.

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multiple testing correction [28] was applied. The raw data is available at NCBI GEO, accession number GSE53795.

RT-PCR analysis

Snap frozen skin biopsies were homogenized in Trisure (Bioline, London, UK) and RNA was extracted according to standard protocols and subjected for cDNA synthesis. Complementary DNA synthesis, and RT-PCR analysis were performed as previously described [29] with 7500 Fast Real Time PCR-system (Applied Biosystems, Foster City, CA) in Finland and Applied Biosystems 7900HT machine in France. The data were analysed by using the $2^{-\Delta\Delta CT}$ method, according to the manufacturer’s instructions. Statistical analyzes were done with Student’s t-test or when variances were significantly different, with nonparametric Mann-Whitney U test using GraphPad Prism software (GraphPad Software Inc., LaJolla, CA).

Cytokine profiling by Luminex technology

Cytokines were extracted from non-lesional and lesional skin which were crushed manually for 4 minutes with a glass potter (Dominique Dutcher S.A, Brumath, France) in 200 µL ice-cold PBS containing Triton X100 0, 2% and protease inhibitors (cOmplete, Mini, EDTA-free) (Roche Applied Science, Mannheim, Germany). Samples were stirred for 30 minutes at 4 °C, at 1400 RPM and then centrifuged for 5 minutes at 4°C, at 10 000 RPM. Supernatants were harvested and the concentration of proteins was determined by colorimetric method (Bio-Rad DC Kit)(Bio-Rad Laboratories, Hercules CA). Cytokines were quantified in duplicate using following Luminex assays (Life Technologies, Carlsbad, CA): Human Cytokine, Premixed 23 Plex, Immunoassay Procarta kit and Milliplex Human Cytokine, Premixed 42 Plex, Immunoassay kit (Merck Millipore, Billerica, MA). Cytokine quantitivities were normalized to the total concentration of protein.

Histology and immunohistochemistry

Biopsies for histological analysis were fixed in 10% buffered formaldehyde. After dehydration/impregnation and inclusion in paraffin, 4 µm-thick serial sections were prepared. Slides were stained using Hematoxylin-Eosin.

For immunohistochemistry of German patient samples, deparaffinised sections were treated for antigen retrieval in 10 mM citrate buffer, pH 6.0 at 98°C for 20 minutes. Mouse antihuman CD3 (clone Ab-9, Thermo Scientific, Fremond, CA) was used to detect T-lymphocytes. IL-17A was detected on the same section with a goat polyclonal antihuman IL-17A antibody (R&D systems, Minneapolis, MN). CD3 was detected with Alexa fluor 594 conjugated antibody and IL-17A was revealed after amplification (Biotin/ Streptavidin FITC). Positive cells were counted visually on slides scanned with the NanoZoomer (Hamamatsu, Japan).

For immunohistochemistry of Finnish samples, heat-induced antigen retrieval of the deparaffinised sections was performed in a microwave oven. Endogenous peroxidase activity was neutralized. The sections were incubated with monoclonal mouse anti-human
antibodies to CD4 (clone 4B12, 1:50, Novocastra, Newcastle, UK), CD8 (clone 4B11, 1:200, Novocastra), CD68 (clone KP-1, 1:10000, Dako, Copenhagen, Denmark), CD83 (clone 1H4b, 1:25, Dako), Foxp3 (clone 236A/E7, 1:100, Abcam Ltd., Cambridge, UK), T-bet (clone 4B10, 1:200, Abcam Ltd.) and goat polyclonal anti-human antibody to IL-17A (1:100, R&D systems, Minneapolis, MN). Bound antibodies were detected using the NovoLink Polymer detection system (Leica Biosystems, Newcastle, UK), the EnVision system (Dako) or Goat-HPR-Polymer kit (Biocare Medical, Concord, CA). Deaminobenzidine (DAB) was used as the chromogen and hematoxylin as the counterstain. Digital image analysis was used to count the positive cells. The digital images were obtained under 20× magnification from all sections with the use of a Nikon Eclipse E600 microscope and a Olympus DP25 digital camera. Images were taken from upper dermal area and around pilosebaceous follicles, resulting in 2 to 7 images per slide. Image analysis was performed as described previously using ImageJ software [30] to record the average number of the positively stained cells per 20× field image (0.14 mm²). Data are presented as median positive cell count (interquartile range). The statistical analysis was performed by IBM SPSS Statistics 19 (IBM, Chicago, IL). Mann-Whitney U test two-tailed p-value less than 0.05 was considered statistically significant.

Results
Genome-wide transcriptome reveals Th17-related signaling significantly elevated in acne lesions
The study was performed in two separate patient cohorts from Germany and Finland (Tables 1 and 2). The gene expression in skin biopsies of the subjects recruited in Germany was screened using Affymetrix microarray technology. In the array analyses, a total of 904 genes were differentially expressed in acne lesions compared with non-lesional skin, and 509 of these were upregulated, whereas 395 were downregulated (Tables 3 and 4).

Table 3. Top 20 up-regulated genes in the German acne patient group based on Affymetrix microarray technology.

| ProbeSet | Gene symbol | Title | Mean expression (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR |
|----------|-------------|-------|-------------------------------------|---------------------------------------------|-----|
| 232170_at | S100A7A     | S100 calcium binding protein A7A | 235 | 29 | <0.001 |
| 202859_x_at | IL8         | interleukin 8 | 162 | 25 | <0.001 |
| 207356_at | DEF4        | defensin, beta 4 | 451 | 25 | <0.001 |
| 211906_s_at | SERPINB4  | serpin peptidase inhibitor, clade B (ovalbumin), member 4 | 768 | 19 | <0.001 |
| 20475_at  | MMP1        | matrix metalloproteinase 1 (interstitial collagenase) | 124 | 19 | <0.001 |
| 212768_s_at | OLFM4      | olfactomedin 4 | 65 | 16 | 0.0018 |
| 205828_at | MMP3        | matrix metalloproteinase 3 (stromelysin 1, progelatinase) | 79 | 16 | <0.001 |
| 204470_at | CXCL1       | chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) | 51 | 15 | <0.001 |
| 210873_x_at | APOBEC3A  | apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A | 53 | 14 | <0.001 |
| 205663_at | S100A12     | S100 calcium binding protein A12 | 47 | 14 | <0.001 |
| 203691_at | PI3         | peptidase inhibitor 3, skin-derived | 987 | 13 | <0.001 |
| 209075_s_at | SPP1       | secreted phosphoprotein 1 | 96 | 13 | <0.001 |
| 206561_s_at | AKR1B10    | aldo-keto reductase family 1, member B10 (aldose reductase) | 135 | 13 | <0.001 |
| 205681_at | BCL2A1      | BCL2-related protein A1 | 81 | 13 | <0.001 |
| 206336_at | CXCL6       | chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2) | 13 | 11 | <0.001 |
| 209774_x_at | CXCL2      | chemokine (C-X-C motif) ligand 2 | 54 | 11 | <0.001 |
| 204580_at | MMP12       | matrix metalloproteinase 12 (macrophage elastase) | 67 | 11 | <0.001 |
| 205114_s_at | CCL3/CCL3L1/CCL3L3 | chemokine (C-C motif) ligand 3/3 chemokine (C-C motif) ligand 3-like 1/chemokine (C-C motif) ligand 3-like 3 | 65 | 10 | <0.001 |
| 210146_x_at | LILRB2      | leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2 | 32 | 10 | <0.001 |
| 210413_x_at | SERPINB3/SERPINB4 | serpin peptidase inhibitor, clade B (ovalbumin), member 3/serpin peptidase inhibitor, clade B (ovalbumin), member 4 | 1936 | 10 | <0.001 |

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accession number GSE53795. A two-component Non Negative Matrix Factorization (NMF) model [32] was applied and allowed a clear separation between lesional and non-lesional acne samples (Figure 2). Bioinformatic analysis of differentially modulated genes using Metacore software identified the inflammatory and immune responses as the main enriched biological processes (Figure 3a). Interestingly, the IL-17 signaling pathway and the Th17-derived cytokines network were ranked fourth in the enriched GeneGo pathways and networks, respectively (Figures 3b and 3c).

| ProbeSet | Gene symbol | Title                                                                 | Mean expression (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR  |
|----------|-------------|----------------------------------------------------------------------|-------------------------------------|---------------------------------------------|------|
| 204515_at | HSD3B1      | hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 | 2897                                | −8.8                                        | 0.002|
| 205380_at | PDZK1       | PDZ domain containing 1                                              | 1914                                | −6.4                                        | 0.002|
| 210576_at | CYP4F8      | cytochrome P450, family 4, subfamily F, polypeptide 8                | 1305                                | −6.3                                        | 0.002|
| 241412_at | BTC         | betacellulin                                                        | 791                                 | −6.1                                        | <0.001|
| 234513_at | ELOVL3      | elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3 | 8094                                | −6.1                                        | 0.003|
| 1553583_a_at | THRSP    | thyroid hormone responsive (SPOT14 homolog, rat)                    | 5008                                | −5.9                                        | 0.003|
| 206143_at | SLC26A3     | solute carrier family 26, member 3                                  | 166                                 | −5.7                                        | <0.001|
| 234980_at | TMEM56      | transmembrane protein 56                                            | 3401                                | −5.4                                        | 0.002|
| 220801_s_at | HAO2     | hydroxoyacid oxidase 2 (long chain)                                 | 1120                                | −5.3                                        | <0.001|
| 230573_at | SGK2        | Serum/glucocorticoid regulated kinase 2                             | 495                                 | −5.3                                        | 0.0016|
| 207958_at | UGT2A1/UGT2A2 | UDP glucuronosyltransferase 2 family, polypeptide A1/UDP glucuronosyltransferase 2 family, polypeptide A2 | 351                                 | −5.0                                        | <0.001|
| 206465_at | ACSBG1      | acyl-CoA synthetase bubblegum family member 1                       | 8201                                | −4.9                                        | 0.002|
| 208962_s_at | FADS1    | fatty acid desaturase 1                                             | 5240                                | −4.8                                        | 0.003|
| 205029_s_at | FABP7     | fatty acid binding protein 7, brain                                 | 4530                                | −4.8                                        | 0.011|
| 239108_at | FAR2        | Fatty acyl CoA reductase 2                                           | 6891                                | −4.7                                        | 0.002|
| 210377_at | ACSM3       | acyl-CoA synthetase medium-chain family member 3                    | 591                                 | −4.5                                        | 0.003|
| 206714_at | ALOX15B     | arachidonate 15-lipoxygenase, type B                                | 6853                                | −4.5                                        | 0.003|
| 221142_s_at | PECR      | peroxisomal trans-2-enoyl-CoA reductase                             | 1668                                | −4.5                                        | 0.0012|
| 236175_at | TRIM55      | tripartite motif-containing 55                                      | 184                                 | −4.4                                        | <0.001|
| 208331_at | BPY2        | basic charge, Y-linked, 2                                           | 1084                                | −4.4                                        | 0.007|

Figure 2. 2-components NMF (Non Negative Matrix Factorization) model. The heatmap of 904 regulated genes on subjects-corrected log2-expressions (legend color blue for low expression and red for high expression). doi:10.1371/journal.pone.0105238.g002
The Affymetrix transcriptome results were confirmed with real-time PCR (RT-PCR). Among the upregulated genes were IL-23, IL-6 and TGF-β, which all are critically involved in Th17 cell activation and differentiation. Furthermore, Th17 cell products IL-17A, IL-22, IL-26, TNF and IL-17 induced chemokines CSF2 and CCL20 were also expressed at higher levels in acne lesions (Table 5). The expression of the upregulated genes of Th17 lineage inducing cytokines IL-1β, IL-6, TGF-β and IL-23 were found significantly increased also in the Finnish cohort by RT-PCR (Figure 4) as well as Th17 signaling cytokine IL-17A.

The increased production of Th17 related cytokines in the acne lesions was confirmed at the protein level by cytokine profiling of manually crushed skin biopsies from German patients by Luminex technology (Table 6). Furthermore, significantly higher numbers of IL-17A positive (IL-17A+) cells were detected by immunohistochemistry both in the papillary dermis and around sebaceous follicles in acne lesions of Finnish patients (Table 7). IL-17A+ cells were scattered in the superficial and deep layers of the dermis (Figure 5a). IL-17A+ cells were mainly lymphocytes but some of them were neutrophils and even mast cells (data not shown). IL-17A+ cells were also identified in the inflammatory infiltrates in the German patient group (Figure 5b). Strongly IL17-positive cells were mainly composed of CD3+ lymphocytes in both lesional and non-lesional samples.

Thus, the upregulation of Th17 pathway found with global Affymetrix analysis was confirmed with RT-PCR and also on the protein level with Luminex and immunohistochemistry in patient samples from Germany. Moreover, the result was similar in a separate patient group from Finland investigated by RT-PCR and immunohistochemistry. While IL-17A in addition to Th17 cells can also be secreted by other CD3+ lymphocytes, including CD8+ cytotoxic T-cells (Tc17) and γδ T cells, the concomitantly observed inductions of Th17-activating and Th17-related cytokines and chemokines observed in two independent patient populations support the observation of Th17 pathway activation in acne lesions.

Innate immune responses were significantly increased in acne lesions

We wanted to investigate also innate immune responses in acne lesions. P. acnes triggers inflammatory responses via TLR2 or Nod-like receptor 3 (NLRP3) -inflammasome, leading to the production of IL-6, and IL-8 by keratinocytes and sebocytes and IL-1β, IL-12 and IL-8 by macrophages and primary monocytes, respectively [15,16,33–35]. The expression of TLR2 and TLR4 were significantly elevated in the acne lesions in the Finnish group (Figure 6a). Of note, TLR2, but not TLR4, was increased in psoriasis control samples as found earlier by Begon et al [36]. The
Table 5. Expression at the mRNA level of markers and cytokines characterizing the Th17 cell subtype in the German acne cohort.

| Th17 signalling         | Markers and cytokines | Affymetrix data                  | RT-PCR                  |
|-------------------------|-----------------------|----------------------------------|-------------------------|
|                         | Gene symbol | Title                           | Mean expression (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR (involved vs. non-involved skin) | Mean CT (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR (involved vs. non-involved skin) |
| **Th17 activation**     | IL23        | interleukin 23, alpha subunit p19 | 95                      | 1.3               | 0.001                          | 33                      | 6.2               | <0.001                          |
|                         | TGFβ1       | transforming growth factor, beta 1 | 111                     | 1.8               | <0.001                         | 24                      | 1.1               | 0.08                           |
|                         | IL6         | interleukin 6                    | 73                      | 4.7               | 0.001                          | 30                      | 6.2               | <0.001                         |
|                         | STAT3       | signal transducer and activator of transcription 3 | 962 | 1.9 | 0.001                          | 22                      | -1.4              | 0.25                           |
| **Th17 related cytokines and chemokines** | IL17A       | interleukin 17A                  | 13                      | 4.3               | 0.01                           | 32                      | 5.6               | <0.001                         |
|                         | IL17F       | interleukin 17F                  | 35                      | 2.5               | 0.01                           | Not evaluated           |                                  |                                 |
|                         | IL22        | interleukin 22                   | 9                       | 1.8               | 0.11                           | 34                      | 2.4               | <0.001                         |
|                         | IL6         | interleukin 6                    | 73                      | 4.7               | 0.001                          | 30                      | 6.2               | <0.001                         |
|                         | TNF         | tumor necrosis factor            | 80                      | 1.6               | 0.003                          | 28                      | 1.1               | 0.31                           |
|                         | IL26        | interleukin 26                   | 24                      | 2.0               | 0.002                          | 31                      | 1.5               | 0.004                          |
|                         | CCL20       | chemokine (C-C motif) ligand 20   | 146                     | 3.3               | 0.002                          | 29                      | 2.4               | <0.001                         |
|                         | IL21        | interleukin 21                   | Not detected            |                  |                                | 34                      | 1.3               | 0.14                           |
| **Receptors**           | IL23R       | interleukin 23 receptor          | Not detected            |                  |                                | 34                      | -1.6              | 0.10                           |
|                         | CCR6        | chemokine (C-C motif) receptor 6 | 91                      | 1.2               | 0.33                           | 28                      | -1.8              | <0.001                         |

Abbreviations: CT, cycle threshold value; FDR, False discovery rate. doi:10.1371/journal.pone.0105238.t005
proinflammatory cytokines IL-1β, IL-6, TNF-α and the chemokine IL-8 were all expressed at a higher level in acne lesions in the Finnish cohort (Figure 4, Figure 6b). Similarly, TLR2 and TLR4 as well as IL-8, IL-6, and TNF-α, were induced in acne lesions in the German group (Table 8).

The expression of antimicrobial peptides (AMPs) was also increased in the acne lesions. AMPs are evolutionary conserved, positively charged molecules that bind membranes of microbes by their hydrophobic surfaces and form pores on the membranes and limit the survival of commensals and potential pathogens also with other mechanisms [37]. The expression of AMPs human cathelicidin antimicrobial protein (hCAP18), lipocalin (LCN2), human beta-defensin 2 (hBD2), hBD3, S100A7 and S100A9 were found significantly upregulated in acne lesions in the Finnish patient group by RT-PCR (Figure 7). Similar findings were observed in German patients (Table 9).

**Figure 4. Th17 related cytokines were increased in acne lesions.** The skin biopsies were obtained from healthy volunteers normal skin (NS), from acne patients non-lesional skin (A NS) and lesional skin (AL) and from psoriasis patients non-lesional skin (P NS) and lesional skin (PL). The expression levels of cytokines were determined by RT-PCR. The expressions of inflammatory cytokines TGF-β, IL-6, IL-1β and IL-23 were increased significantly both in acne and psoriasis but IL-1β and TGF-β expression was higher in acne. IL-23 was increased more in psoriasis lesions. IL-17A was significantly expressed in lesional acne and psoriasis skin. IL-22, was slightly elevated in acne and psoriasis, however significantly more in psoriasis. *P<0.05, **P<0.01, ***P<0.001; bars represent mean ±SEM. RU, relative units; TGF-β, transforming growth factor- beta; Th17, T helper type 17.

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**Th1 and T regulatory cell markers were elevated at the RNA level in acne lesions**

In addition to the Th17 pathway, we examined other T-cell subsets in acne lesions. Mouser and co-workers [38] demonstrated a subpopulation of Th1 cells generated from early inflamed acne lesions, but regulatory T cells (Tregs) are not investigated in acne lesions. IL-12 is a pivotal cytokine in activating Th1 responses, and is induced in monocytes by *P. acnes* via TLR2 [6] and in vitro in PBMCs [20]. The IL-12 subunit p40, but not p35, was found significantly increased in both acne (P<0.01) and psoriasis (P<0.001) by RT-PCR (Figure 8). Furthermore, the Th1 polarizing key transcription factor T-bet, Th1 effector cytokine IFN-γ, and Th1 type chemokine receptor CXCR3 were also expressed at a higher level in both acne and psoriasis lesions, indicating the involvement of Th1 effector cells (Figure 8). Similar findings were observed in the German cohort, since STAT1 a positive regulator
of Th1 development was induced, and the CXCR3 ligands CXCL9, CXCL10 and CXCL11, were overexpressed in lesional acne skin (Table 10).

Foxp3 is a specific marker of natural and induced Tregs, which are efficient suppressors of both innate and adaptive immune responses. TGF-β and IL-10 are involved in the induction and function of Tregs [39]. In the Finnish cohort, the expression of Foxp3, TGF-β and IL-10 were found significantly up-regulated in acne lesions by RT-PCR, and the latter two were at higher levels in acne lesions compared with psoriatic lesions (Figure 8). In the German cohort, TGF-β and IL-10 displayed a slightly enhanced expression in acne lesions and also the expression of Foxp3 was detected (Table 11).

Th2 cells produced cytokines IL-4, IL-5 and IL-13 were either not detected or not modulated in the German cohort by array technology, and detected at a very low level in the Finnish cohort by RT-PCR, suggesting that Th2 cells are not involved in the inflammatory response in acne and in psoriasis (data not shown).

Cells of adaptive immunity were recruited to acne lesions

In addition to measurement of gene expression in skin samples, we investigated the infiltration of key cell types in acne lesions. Apart from IL-17A, skin biopsy tissue sections were stained for CD4, CD8, T-bet, Foxp3, CD68 and CD83. All sections of lesional and non-lesional acne samples contained immune cells in papillary dermis and around sebaceous follicles. The most common cells were CD68+ macrophages and CD4+ T-cells. The count of CD4+ cells and CD83+ mature dendritic cells was significantly higher both in the papillary dermis and around follicles in acne lesions, when compared with non-lesional skin. (Figure 5e–h, Table 7). T-bet+ cells were also detected both in lesional and non-lesional skin of acne patients, but at significantly higher numbers in acne lesions, both in the papillary dermis and around follicles (Table 7).

Table 6. Expression at the protein level of markers and cytokines characterizing the Th17 cell subtype in the German acne cohort.

| Th17 Signalling | Symbol | Title | Protein level pg/mg (non-involved skin) | Fold change (involved vs. non-involved skin) | P-value (involved vs. non-involved skin) |
|----------------|--------|-------|----------------------------------------|---------------------------------------------|----------------------------------------|
| Th17 activation | IL23   | interleukin 23, alpha subunit p19 | 0.3 | 3.1 | 0.17 |
|                | IL6    | interleukin 6 | 1.1 | 15.0 | <0.001 |
| Th17 related cytokines and chemokines | IL17A  | interleukin 17A | 1.1 | 3.8 | 0.01 |
|                | IL17F  | interleukin 17F | 0.3 | 4.9 | <0.001 |
|                | IL22   | interleukin 22 | 4.6 | 3.1 | 0.03 |
|                | IL6    | interleukin 6 | 1.1 | 15.0 | <0.001 |
|                | CCL20  | chemokine (C-C motif) ligand 20 | 1.1 | 2.5 | 0.02 |
|                | IL21   | interleukin 21 | 7.5 | 3.9 | 0.01 |
|                | CSF2   | colony stimulating factor 2 | 1.1 | 2.2 | 0.01 |

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Table 7. Number of positively stained cells in lesional and non-lesional skin of acne patients (n = 16) as assessed by immunohistochemistry.

| Positive-cell counts² in upper dermis | Positive-cell counts² around follicles |
|--------------------------------------|---------------------------------------|
| Lesional | Non-lesional | P-value | Lesional | Non-lesional | P-value |
| CD4 | 33 (22–52.8) | 16.5 (9.3–26.3) | **0.007** | 52 (44–127.5) | 8 (4–14.5) | 6.5 × 10⁻⁵ |
| CD8 | 6 (2.3–15.3) | 3 (1.3–9.5) | **0.225** | 11 (4.5–18.5) | 1 (1–2) | 8.5 × 10⁻⁵ |
| T-bet | 6 (3–17) | 2.5 (1–3) | **0.005** | 16.5 (3.5–31.5) | 0 (0–1.8) | 8.6 × 10⁻⁵ |
| IL-17A | 4.5 (2.3–8) | 2 (2–3) | **0.030** | 6 (3.5–9.3) | 3 (1.3–5.3) | **0.023** |
| Foxp3 | 4.5 (2–9.8) | 1 (1–3) | **0.003** | 6 (0.8–13) | 1 (1–2) | **0.072** |
| CD68 | 38 (18.3–68) | 5.5 (1–13) | **3.1 × 10⁻⁵** | 77.5 (27–131.5) | 3.5 (2.3–5.8) | 2.6 × 10⁻⁵ |
| CD83 | 7 (2.3–13) | 1.5 (0.3–2.8) | **1.5 × 10⁻⁴** | 12 (4.5–27) | 1 (0–2) | 2.3 × 10⁻⁶ |

Abbreviations: IHC, immunohistochemistry; IQR, interquartile range.

16 of total 20 acne patients samples in Finnish patient cohort were examined by IHC.

²Positive-cell counts per field image are expressed as medians (IQR). Statistically significant difference of P<0.05 in the lesional acne skin compared to non-lesional skin. Significant P values are bold.

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around follicles, demonstrating the presence of Th1 cells (Figure 5c, Table 7). Yet, the number of Foxp3+ cells was significantly higher in the acne lesions in the papillary dermis but not around follicles (Figure 5d, Table 7). Finally, the count of CD8+ T-cells was significantly higher in infiltrates around follicles in lesional acne (Table 7).

**Discussion**

In the current study we examined the inflammatory reaction and especially the IL-23/Th17/IL-17A axis in lesions of acne in vivo. Our results in two independent groups of acne patients show that the cytokines involved in the differentiation of Th17 cells and the expression of the main effector cytokines IL-17A and IL-17F were significantly expressed in lesions of acne at mRNA and protein level.
IL-17A and IL-17F are key cytokines for the recruitment and activation of neutrophils and can target different cell types including keratinocytes, endothelial cells, monocytes, fibroblasts to induce pro-inflammatory mediators IL-6, TNF-α, IL-1β, PGE2, nitric oxide, matrix metalloproteinases and chemokines (GM-CSF, G-CSF, CXCL1, CXCL8, CCL2, CCL7, CCL20) [40]. In psoriasis IL-17A, IL-17F, IL-22 and IL-20 act on keratinocytes leading to epidermal hyperplasia [41,42]. IL-22, which is secreted by both Th17 and Th22 cells [40], induces the production of IL-20 [43]. In our study elevated levels of IL-22 (Figure 4, Table 5) and IL-20 (Figure 6c) were found in acne lesions by RT-PCR. IL-17 and IL-22 induces the production of AMPs [44]. The expression of AMPs was up-regulated in acne, which confirms prior observations [23,26,45–48]. Probably due to the high level of

![Figure 6. The expression of proinflammatory mediators in Finnish patient cohort examined by RT-PCR.](https://example.com/figure6.png)
AMPs, acne and psoriasis are seldom complicated by secondary infections.

We found increased expressions of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and TGF-β that are also important in Th17 cell activation. All these cytokines enhance generation of Th17 cells from naive T-cells with activated dendritic cells in the presence of IL-23 [40]. TGF-β is a critical cytokine in both Th17 and Treg-cell differentiation. TNF-α can synergize with IL-17 to promote inflammation in psoriasis and also IL-17 and IFN-γ synergize in pro-inflammatory cytokine production in keratinocytes [49], [50]. On the contrary to Agak et al [20], we found also increased expression of Th17 cells stabilizing cytokine IL-23p19 both at the RNA and protein level in vivo. IL-23 is secreted by activated dendritic cells and macrophages and situations in vitro

### Table 8. Expression at the mRNA level of proinflammatory mediators in the German acne cohort determined by Affymetrix microarray technology.

| Gene symbol | Title                  | Mean expression (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR (involved vs. non-involved skin) |
|-------------|------------------------|-------------------------------------|---------------------------------------------|------------------------------------|
| TLR2        | toll-like receptor 2   | 682                                 | 1.9                                          | 0.006                              |
| TLR4        | toll-like receptor 4   | 126                                 | 2.1                                          | 0.0006                             |
| IL6         | interleukin 6          | 73                                  | 4.7                                          | 0.001                              |
| TNF         | tumor necrosis factor  | 80                                  | 1.6                                          | 0.003                              |
| IL8         | interleukin 8          | 162                                 | 24.7                                         | 0.0001                             |

Abbreviations: FDR, false discovery rate.
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**Figure 7. Antimicrobial peptides are increased in acne and psoriasis lesions by RT-PCR.** The expressions of mRNAs were significantly higher in psoriasis except for hCAP-18, which was similarly expressed in both. *P<0.05, **P<0.01, ***P<0.001; bars represent mean ±SEM. BD2, human beta-defensin 2; BD3, human beta-defensin 3; hCAP-18, human cathelicidin antimicrobial protein 18; LCN2, lipocalin-2; RU, relative units. doi:10.1371/journal.pone.0105238.g007
Table 9. Expression at the mRNA level of antimicrobial peptides in the German acne cohort determined by Affymetrix microarray technology.

| Gene symbol | Title                                           | Mean expression (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR (involved vs. non-involved skin) |
|-------------|-------------------------------------------------|-------------------------------------|---------------------------------------------|-------------------------------------|
| CAMP        | cathelicidin antimicrobial peptide              | 121                                 | 1.5                                         | 0.004                               |
| DEFB103A    | defensin, beta 103B                             | 117                                 | 1.9                                         | 0.0001                              |
| DEFB4A      | defensin, beta 4A                               | 451                                 | 24.6                                        | 0.0001                              |
| LCN2        | lipocalin 2                                     | 1 998                               | 2.6                                         | 0.004                               |
| S100A7      | S100 calcium binding protein A7                 | 7 058                               | 4.1                                         | 0.0004                              |
| S100A9      | S100 calcium binding protein A9                 | 4 086                               | 5.9                                         | 0.0003                              |

Abbreviations: FDR, false discovery rate. doi:10.1371/journal.pone.0105238.t009

Th17 Pathway in Acne
and in vivo are different. Our results are in line with the prior observations concerning up-regulated TNF-α and IL-1β in acne lesions examined by RT-PCR [25]. Recently, P. acnes is identified as a trigger of monocyte-macrophage NLRP3- inflammasome activation and IL-1β processing and secretion, and further as a trigger of an innate immune response in the skin [15]. The expression of cytokines IL-6, IL-12p40, INF-γ and TGF-β have not been studied earlier in acne lesions as also noticed by Thiboutot and co-workers [19], nor cytokines IL-17A, IL-17F, IL-23p19, IL-22 and chemokines CCL20 and CSF2. The IL-23/Th17 pathway and especially cytokine IL-17A is a key cytokine that activates pathogenic inflammation in psoriasis [51], [52]. We hypothesize that follicular hyperkeratinization, increased expression of AMPs and accumulation of neutrophils in acne lesions may be in part explained by the activation of the IL-23/IL-17 axis (Figure 9).

Previously described CD4+ T cells in early acne lesions have not been determined in details [4,5]. We defined the markers of T cell subsets at mRNA level and studied Th1 (T-bet), Th17 (IL-17A), Treg (Foxp3) cells, cytotoxic CD8+ T-cells and CD68 macrophages and CD63 positive activated dendritic cells in clinically early acne lesions by immunohistochemistry both phenotypically and proportionally (Figure 5, Table 7). Recently Agak and co-workers [20] presented IL-17+ lymphoid cells in three closed comedone-type acne lesions by immunohistochemistry. In addition to Th17 cells IL-17A can also be secreted by CD8+ cytotoxic T-cells (Tc17), γδ and γδ T cells and even neutrophils and mast cells [33]. In our study the observations of IL-17A+ by immunohistochemistry resembled the findings made by Res et al [34] in psoriasis. The cells were few and they were scattered throughout the samples (Table 7, Figure 5a), however, significantly more numerous in lesional areas. IL-17A+ cells were mainly lymphocytes but some of them were neutrophils and even mast cells. Further investigations are needed to examine whether CD8+ cells in acne lesions were either Tc17 or Tc22 cytotoxic cells. To examine early CD4+ reaction, biopsies were taken from small visible developing papules for microarray/RT-PCR and immunohistochemistry. Previously Norris and Cunliffe have described the biopsied 6-72 h lesions had morphology of small papules with a minimal surrounding erythematous flare, in which lymphoid cells outnumbered polymorphonuclear cells (PMNs) in the 6 h biopsies and PMNs became increasingly numerous in the 24-72 h lesions [5]. In our study the biopsied lesions were similar in appearance: comedones with erythematous flare or small papules (Figure 1). The lesions in immunohistochemistry revealed neutrophils in many of the lesions but fewer than lymphoid cells and moreover the rupture of the follicle walls was not usually seen suggesting the biopsied lesions were not fully developed inflammatory lesions.

To best of our knowledge Treg cells have not been examined in acne lesions earlier. We found significantly increased number of Foxp3+ cells in papillary dermis in immunohistochemistry. Tregs prevent autointoimmunity and suppress immune responses. Retinoic acid can regulate reciprocally Tregs and Th17 via TGF-β dependent generation of Foxp3 [35], [36], a mechanism that may be of importance in the treatment of acne by isotretinoin. Elevated serum levels of anti-inflammatory cytokine IL-10 are found in acne patients and the expression of IL-10 is increased in acne lesions [13], [25]. In our study lesional IL-10 mRNA was higher in acne than in psoriasis (Figure 8). Of note, TLR2 recognition of Candida albicans suppresses inflammatory responses via production of IL-10 and enhanced Treg survival [14] but it is not known if TLR2 recognition in acne has the same consequences. Though acne is often a chronic disease, a single acne lesion is seldom secondarily

### Table 10. Expression at the mRNA level of markers and cytokines characterizing the Th1 cell subtype in the German acne cohort determined by Affymetrix microarray technology.

| Gene symbol | Title | Mean expression (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR (involved vs. non-involved skin) |
|-------------|-------|------------------------------------|---------------------------------------------|--------------------------------------|
| CXCL10      | chemokine (C-X-C motif) ligand 10 | 131                                | 3.1                                         | 0.01                                 |
| CXCL11      | chemokine (C-X-C motif) ligand 11 | 24                                 | 2.8                                         | 0.05                                 |
| CXCL9       | chemokine (C-X-C motif) ligand 9  | 131                                | 2.3                                         | 0.04                                 |
| CXCR3       | chemokine (C-X-C motif) receptor 3 | 86                                | 1.1                                         | 0.03                                 |
| STAT1       | signal transducer and activator of transcription 1 | 500                        | 2.2                                         | 0.01                                 |
| TBX21       | T-box 21                           | 65                                 | 1.2                                         | 0                                    |
| IL12B       | interleukin 12B (p40)              | Not detected                       | NA                                          | NA                                   |
| IL12A       | interleukin 12A (p35)              | Not detected                       | NA                                          | NA                                   |
| IFNG        | interferon, gamma                  | Not detected                       | NA                                          | NA                                   |

Abbreviations: FDR, false discovery rate; NA, Not applicable.

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Figure 8. The expression of Th1 cell and Treg markers were activated in acne by RT-PCR. Th1 transcription factor T-bet, Th1 cell surface chemokine receptor CXCR3 and IFN-γ (the effector cytokine of Th1 cells) mRNAs were significantly up-regulated in acne and psoriasis lesions. The transcription factor of Tregs, Foxp3, was elevated in acne and psoriasis. TGF-β which is both Th17 and Treg lineages inducing cytokine was significantly increased in acne lesions. The product of Tregs, immunosuppressive cytokine IL-10 was expressed more in acne than in psoriasis. *P<0.05, **P<0.01, ***P<0.001; bars represent mean ± SEM, RU, relative units; TGF-β, transforming growth factor-β; Th1, T helper type 1; Treg, regulatory T cell.

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infected and is rapidly demarcated. The increased IL-10 expression and Tregs may demarcate the inflammation of a single acne lesion efficiently from the beginning. The reaction soon changes after ruptures of follicular walls into the direction of wound healing and fibrosis [34].

In conclusion, we demonstrated the presence of IL-17A+ cells, which were mainly lymphocytes, in clinically early visible inflamed acne lesions, and also the activation of cytokines, chemokines and antimicrobial peptides known to be typical for the Th17/IL-17 pathway. Th1, Th17 and also CD8+ activation and IL-17 related AMP and CXCL chemokine production with neutrophil attraction in acne lesions could be important factors among others in acne pathogenesis. Further studies will show the relevance of these findings also in the pathogenesis of different variations of severe acne such as acne cystica, acne fulminans and autoinflammatory syndromes with acne symptoms like SAPHO or PAPA syndromes, and indicate new possibilities to target the inflammatory response in acne diseases.

### Table 11. Expression at the mRNA level of markers and cytokines characterizing the Treg cell subtype in the German acne cohort determined by Affymetrix microarray technology.

| Gene symbol | Title                        | Mean expression (non-involved skin) | Fold change (involved vs. non-involved skin) | FDR (involved vs. non-involved skin) |
|-------------|------------------------------|-------------------------------------|---------------------------------------------|-------------------------------------|
| FOXP3       | forkhead box P3              | 62                                  | 1.2                                         | 0.002                               |
| IL10        | interleukin 10               | 31                                  | 1.9                                         | 0.002                               |
| TGFB1       | transforming growth factor, beta 1 | 111                                  | 1.8                                         | 0.001                               |

Abbreviations: FDR, false discovery rate.

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**Figure 9. The effect of IL-17 on keratinocytes.** Th17-cells are defined to secrete IL-17 cytokines. Also some other cells can secrete IL-17 cytokines when properly activated. IL-17A and IL-17F regulate genes on keratinocytes, that are involved in innate immune defence, such as antimicrobial peptides (beta-defensins, S100 proteins A7/9, cathelicidin, LCN2) and a range of chemokines G-CSF, CXCLs and CCL20 which regulate neutrophil and lymphocyte trafficking. IL-17A and IL-17F stimulate keratinocytes leading to keratinocyte proliferation. Also vascular inflammation is activated.

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Supporting Information

Figure S1 A flowchart of the experimental design. The figure clarifies the methods used in the different cohorts of the study.

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Conceived and designed the experiments: H-LK RP NF KT HA AL PM CM UBP JV. Performed the experiments: H-LK SL NF JVP MEK BB BM SD PR. Analyzed the data: H-LK RP NF DG JVP MK HA IC BM SD JVV. Contributed reagents/materials/analysis tools: KT AL HA CM UBP SD. Contributed to the writing of the manuscript: H-LK RP NF KT AL IC BB SD JVV.
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