

Conjunctival inflammation in dupilumab-treated atopic dermatitis comprises a multicellular infiltrate with elevated T1/T17 cytokines: A case series study

To the Editor,

Conjunctivitis is a frequently reported adverse event in atopic dermatitis (AD) patients treated with dupilumab, the first antibody-based treatment for AD targeting the interleukin (IL)-4 receptor- α -subunit.¹ In a recent study, we demonstrated scarcity of intraepithelial goblet cells (GCs) accompanied by a mixed immune-cell infiltrate in conjunctival biopsies from dupilumab-treated AD patients developing conjunctivitis.² In the current case series study, we further specified these infiltrating cells, in order to improve the understanding of this new entity of conjunctivitis.

Conjunctival biopsies from six AD patients (4 male; median age 38.5 years, interquartile range (IQR) 29.0–56.5, median Eczema Area Severity Index (EASI) score before start dupilumab 20.5, IQR 15.4–27.9) with active conjunctivitis developed during dupilumab treatment confirmed by an ophthalmologist (median time from initiation of dupilumab treatment to onset 59.5 days, IQR 51.5–80.5) were evaluated, of which for five histopathology has been described in a previous study² (Table S1a). Conjunctival biopsies of two healthy controls (HCs) were included. Biopsies were histologically assessed, and additionally stained with a panel of 27 metal-conjugated antibodies (Figure 1A, Table S2) for imaging mass cytometry (detailed methods in online supplementary).

Most frequently reported ophthalmological characteristics were tarsal and bulbar conjunctivitis (83.3% and 66.7%, respectively), and blepharitis (83.3%). Median goblet cell density was 2.6 cells/mm (IQR 1.1–4.0) in patients compared to 4.1 cells/mm and 9.8 cells/mm in HCs (Table S1b).

The subepithelial cellular infiltrate in inflamed conjunctival tissue of dupilumab-treated AD patients ranged from limited (patient 2) to moderate (patients 1 and 6) to extensive (patients 3, 4, and 5), and comprised a diverse panel of infiltrating immune cells, including CD4+ and CD8+ T cells (Figure 1B–D), CD11c+ dendritic cells, CD14+ monocytes, and CD68+ macrophages in all patients (Figures S1 and S4). Within the T-cell infiltrates high expression of ICOS, Ki67 and HLA-DR were observed in all patients, indicating an activation state accompanied by local proliferation (Figures S2a and S4). In five patients, CD8+ T cells co-expressed granzyme B, indicating cytotoxic activity (Figures S3 and S4). Significantly increased signals of

IFN γ , TNF α , IL-10, and IL-17A were observed within subepithelial cell infiltrates in all patient samples compared to non-infiltrated reference regions and HCs (Figure 2). Finally, numerous FOXP3+ regulatory T cells were identified in conjunctival infiltrates of patients 1, 3, 4, and 5 (Figures S2b and S4).

The conjunctival epithelium normally is a GC rich tissue, and IL-13 is the predominant cytokine promoting GC proliferation and mucus secretion.³ Various types of dry eye diseases have been associated with GC loss, and decreased GC density has been associated with increased IFN γ expression, and increased numbers of HLA-DR+, CD11c+, and CD45+ inflammatory cells in the conjunctiva.^{3,4} These results are in line with our findings of GC scarcity combined with an highly activated multicellular infiltrate and increased local Th1-related cytokine production in dupilumab-treated AD patients developing conjunctivitis. Based on these findings,² dupilumab might affect GC development and function by inhibiting IL-13, resulting in reduced production of protective mucus and immunoregulatory factors, promoting conjunctival inflammation, which may be further reinforced by the IFN γ -skewing effect of dupilumab through blocking both IL-4/IL-13 signaling in T cells.

The fact that conjunctivitis has only been reported in AD patients who are treated with dupilumab, and not in asthma or chronic rhinosinusitis, might be explained by the high incidence of ocular surface disease in AD and its association with GC cell loss. More severe AD has been associated with lower GC density, implicating that severe AD patients are at higher risk of developing conjunctivitis during dupilumab treatment.⁵

Treatment with ocular cyclosporine A (CsA) emulsion has shown to significantly increase GC density in patients with dry eye syndrome and to reduce conjunctival T-cell infiltration, activation, and cytokine expression of especially IFN γ in atopic keratoconjunctivitis patients.^{6,7} In view of our findings, CsA eye drops and/or other calcineurin inhibitors, such as tacrolimus eye ointment, might have the potential to suppress conjunctival inflammation and restore development and function of GCs in patients developing dupilumab-associated conjunctivitis.

A limitation of this study is the relatively small sample size and the lack of baseline samples before initiation of dupilumab due to the

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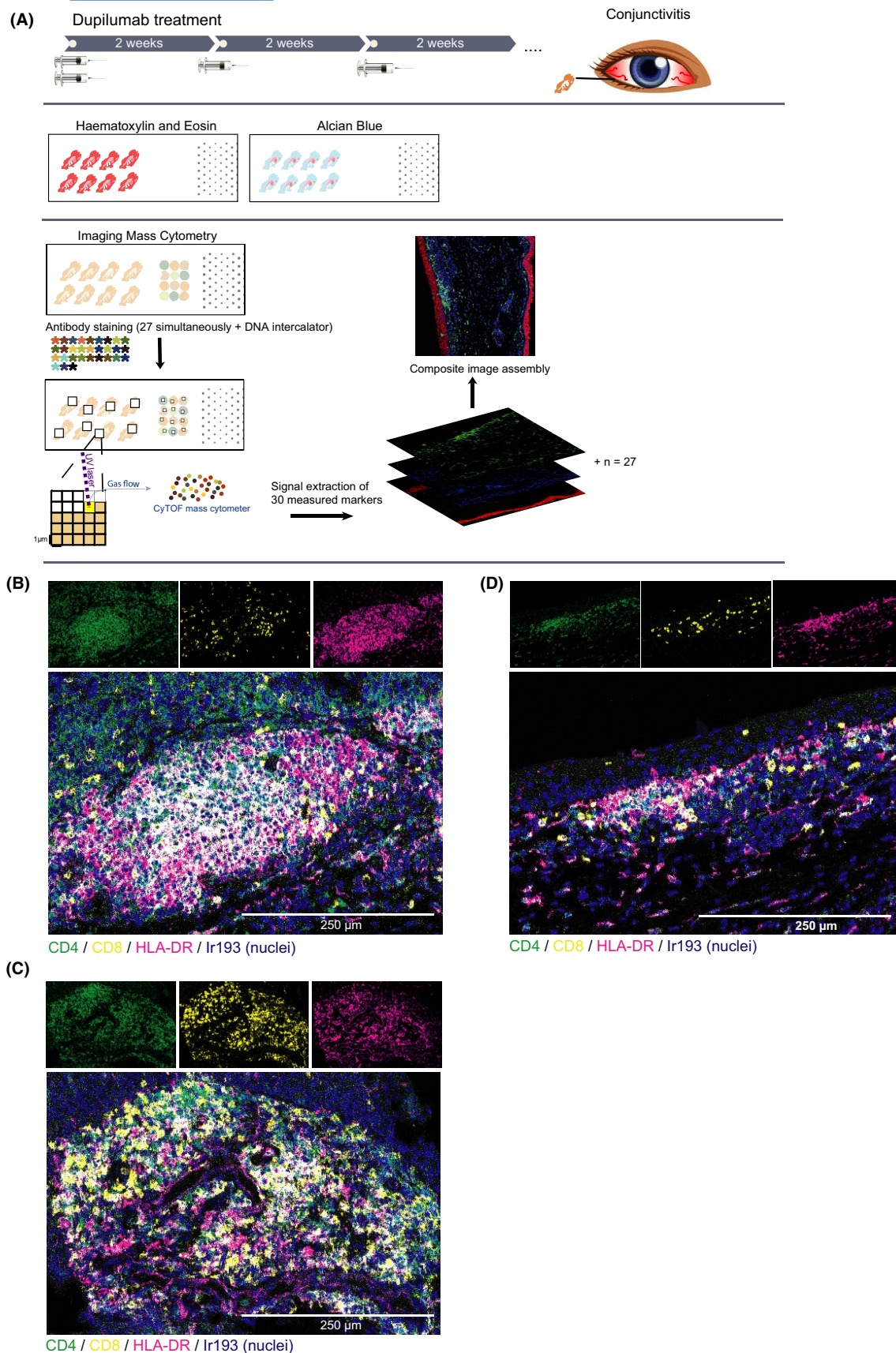
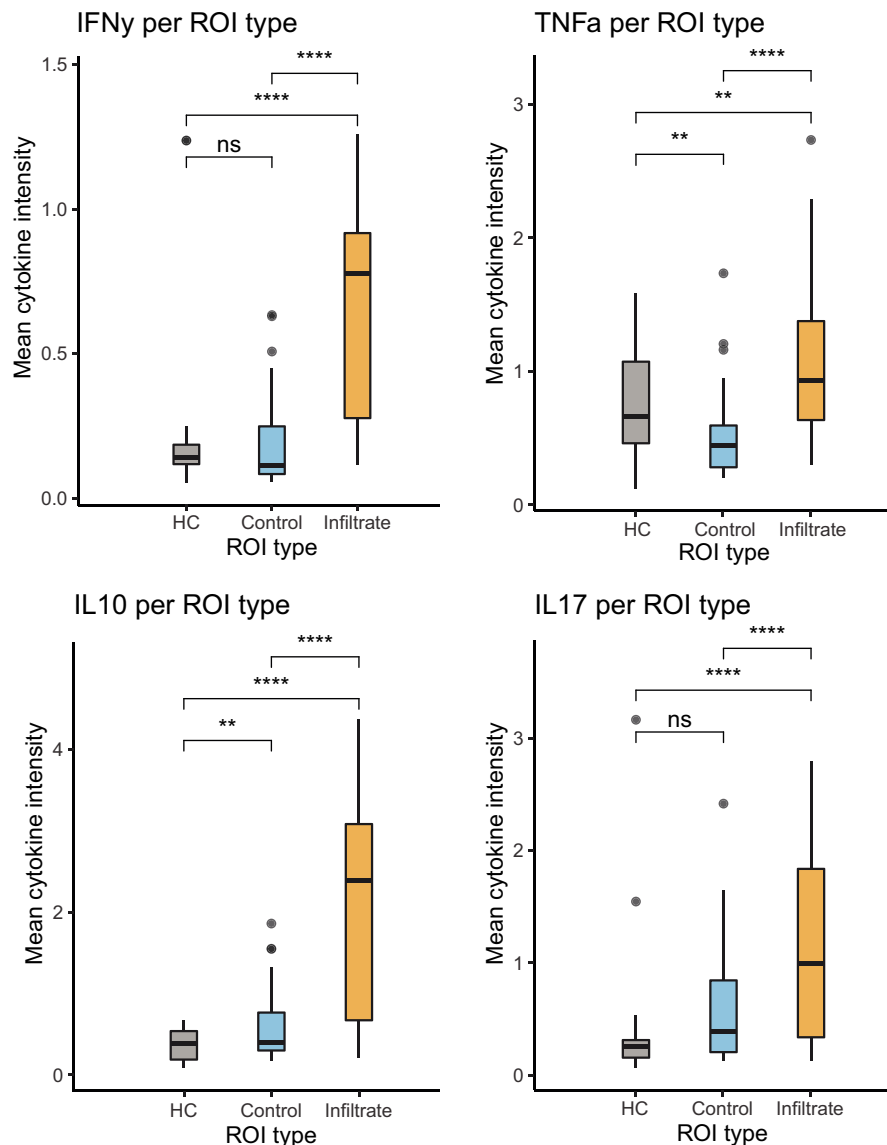


FIGURE 1 Graphical workflow of sample collection, section staining, and Imaging Mass Cytometry. (A). Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from AD patients developing conjunctivitis during dupilumab treatment. Representative images of patient 3 (B), patient 4 (C), and patient 5 (D) showing overlay of CD4 (green), CD8 (yellow), HLA-DR (magenta), and DNA intercalator (Ir 193) (blue)

FIGURE 2 Mean cytokine signal intensity plotted for IFN γ , TNF α , IL-10, and IL17. Mean signal intensities per μm were calculated from three types of region of interest (ROI) within the samples: T-cell infiltrated ROIs from patient samples, non-infiltrated reference ROIs from patient samples, and control ROIs from HC samples, based on composite images including CD4, CD8, CD14, and Ecadherin. Boxes represent medians with first and third quartiles (lower and upper hinges). The upper and lower whiskers extend from the hinge to the largest and smallest value, respectively, no further than 1.5* interquartile range. Significance levels correspond to the following p values: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$



difficulty of patient recruitment for conjunctival biopsies. A further limitation is the lack of specific Th2-related cytokines. Nevertheless, we were able to obtain a clear and consistent characterization of the local conjunctival infiltrate. Lastly, the extent of the conjunctival infiltrate was heterogeneous within the 6 patients, which might be explained by the variety in the duration, severity, and location of the conjunctivitis at the moment of sampling. However, our data show that the inflammatory profile of the infiltrates applies to all six patients.

In conclusion, our findings might indicate that dupilumab-associated IL-4/IL-13 signaling inhibition in combination with increased local Th1-related cytokine production can underlie the loss of GCs and their essential immunomodulatory role in the conjunctiva, hence leading to dry eyes, a highly activated multicellular infiltrate, and tissue damage. In the future studies, longitudinal evaluation of conjunctival GC numbers with less invasive techniques such as conjunctival impression cytology and tear IFN γ concentrations could further confirm this.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

D.S. Bakker reports personal fees from Sanofi/Regeneron, personal fees from Leo Pharma, outside the submitted work. C.M. van Luijk reports personal fees from Sanofi Europe and Santen Europe, outside the submitted work. Dr. de Bruin-Weller reports grants and other from Sanofi-Genzyme, grants and other from Regeneron, grants and other from AbbVie, other from Arena, other from Almirall, grants and other from Eli-Lilly, other from Galderma, other from Janssen, grants and other from Leo Pharma, other from Pfizer, outside the submitted work. Dr. Thijs reports personal fees from Sanofi/Regeneron and Leo Pharma, outside the submitted work. Dr. Vercoulen reports grants from NWO Gravitation 024.001.028 cancergenomicscenter.nl, during the conduct of the study; grants from WKZ fund research grant, grants from TKI-Health Holland grant, grants and non-financial

support from TigaTx B.V., grants from Vrienden van het UMC Utrecht, non-financial support from Life Science editors parental leave grant, outside the submitted work. Dr. van Wijk reports grants from Investigator initiated studies Regeneron and Leo Pharma, outside the submitted work. All other authors have nothing to disclose.

DATA AVAILABILITY STATEMENT

No datasets were generated during this study.

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SUPPORTING INFORMATION

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

SUPPORTING INFORMATION

Conjunctival inflammation in dupilumab-treated atopic dermatitis comprises a multicellular infiltrate with elevated T1/T17 cytokines: a case series study

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SUPPLEMENTAL MATERIALS AND METHODS

Patients

AD patients who developed conjunctivitis during treatment with dupilumab 300mg every other week at the National Expertise Center for Atopic Dermatitis (department of Dermatology and Allergology, University Medical Center Utrecht, the Netherlands) were screened for inclusion. Patients with new onset or worsened conjunctivitis confirmed by an ophthalmologist after starting dupilumab and in whom a diagnostic conjunctival biopsy was performed were eligible for inclusion. Patients were not treated with any ocular anti-inflammatory medication at the moment of biopsy. Ophthalmological characteristics were examined and graded in terms of severity by an experienced ophthalmologist following the Utrecht Ophthalmic Inflammatory and Allergic disease ocular surface score.¹ Clinical data were extracted from an online Good Clinical Practice database called BioDay registry, including a prospective cohort of adult patients with moderate-to-severe AD treated with dupilumab in daily practice. ClinicalTrials.gov identifier: NCT03549416, retrospectively registered June 8, 2018. All patients signed Institutional Review Board-approved written consent, adhering to the Declaration of Helsinki Principles.

Biopsy collection

Topical local anesthetic with 0.4% oxybuprocaine hydrochloride and 0.5% tetracaine was applied to the conjunctival surfaces. After topical anesthetic, the lower eyelid was retracted and the peripheral inferior bulbar conjunctiva was grasped with forceps. A strip of approximately 3mm in diameter from the bulbar conjunctiva near the inferior fornix was excised using Westcott scissors. Bulbar conjunctival biopsies of two healthy controls (HCs), obtained during corneal transplantation due to endothelial dysfunction were included as comparison.

Sample slides

Conjunctival biopsies were fixed in 10% neutral buffered formalin, paraffin-embedded, and three slides containing consecutive 5 µm-thick sections of all samples were prepared. One slide was stained with haematoxylin and eosin (HE) for histological assessment and another with Alcian blue for identification of goblet cells (GCs). A third slide was stained for imaging mass cytometry (IMC)(Figure 1a). IMC is an emerging imaging technology that combines immunohistochemistry with high-resolution laser ablation of stained tissue sections followed by CyTOF mass cytometry. This approach enables sharp-contrast imaging of up to 37

proteins simultaneously at a subcellular resolution.² In addition to conjunctival biopsy sections of patients and controls the sample slide for IMC analysis contained a Tissue Micro Array (TMA) section. The TMA consisted of cores from ten different tissues (tonsil, breast, placenta, skin, liver, lung, small intestine, ovarian, spleen, colon). In this way, each antibody could be positively validated on tissues known to express the target antigen and negatively validated on tissues known to be negative for that antigen.

Imaging Mass Cytometry

Antibodies and metal conjugation

All antibodies in the panel were initially tested by immunofluorescent staining on *Formalin-Fixed Paraffin-Embedded* (FFPE) sections of tonsil or spleen (depending on anticipated epitope abundance) and colon. Next, the purified carrier-free IgG or polyclonal antibodies were conjugated to lanthanide metals (Fluidigm, San Francisco, CA, USA) (Supplementary Table E1) using the MaxPar antibody labeling kit and protocol (Fluidigm). After conjugation, all antibodies were eluted in antibody stabilization buffer (Candor Bioscience) to obtain a final concentration of 0.5 mg/ml and stored at 4°C. Before sample slide hybridization, all antibodies were tested by IMC analysis of tonsil or spleen and colon to ensure that antibody specificity was not affected by conjugation and to determine the optimal antibody concentration.

Staining

The slide was baked for 1.5 hours at 60°C, deparaffinized with fresh xylene for 20 min and subsequently rehydrated in descending grades of ethanol (100% (10 min), 95%, 80%, 70% (5 min each)). After washing for 5 min in milliQ and 10 min in PBST (PBS containing 0.1% Tween-20), heat-induced epitope retrieval was conducted in Tris/EDTA (10 mM/1 mM, pH 9.5) for 30 min in a 95°C water bath. The slide was allowed to cool to 70°C before washing in PBST for 10 min. To decrease non-specific antibody binding, tissue sections were blocked with 3% BSA and Human TruStain FcX (1:100, BioLegend) in PBST for 1 hour at RT. The antibody cocktail was prepared by mixing all antibodies at concentrations specific for the assay in PBST+0.5% BSA. After careful removal of the blocking buffer, the slide was incubated overnight at 4°C with the antibody cocktail. Following three 5 min washes in PBST and rinsing in milliQ the tissue was counterstained with 0.1% toluidine blue for 5 min to

enable tissue structure visualization under bright field microscopy if desired. Upon washing for 5 min in milliQ, the slide was incubated with Ir-intercalator (1:500 in PBST, Fluidigm) for 60 min at RT. Finally, the slide was washed in milliQ and air dried at least for 20 min at RT.

High-spatial resolution laser ablation of tissue sections

Images were acquired at a resolution of 1 μm using a Hyperion Imaging System (Fluidigm). Regions of interest (ROIs) were selected based on hematoxylin and eosin stains performed on consecutive sections after which areas with approximate size of $1,000 \times 1,000 \mu\text{m}$ for conjunctival samples and $500 \times 500 \mu\text{m}$ for TMA samples were ablated and acquired at 200 Hz.

Image visualization

Pseudo-colored intensity maps were generated of each mass channel. Composite images were created for each sample using Image J (version 1.47), and any changes to the brightness or contrast of a given marker were consistent across all samples.

Cytokine signal intensity

To compare cytokine expression, three types of ROIs were selected within the samples: T-cell infiltrated ROIs from patient samples, non-infiltrated reference ROIs from patient samples, and control ROIs from HC samples. ROIs including blood vessels were excluded (based on H&E and morphology). A maximum of 10 ROIs of equal size ($60 \times 90 \mu\text{m}$) per type per patient were selected based on composite images including CD4, CD8, CD14 and Ecadherin. The mean signal intensity per μm^2 was calculated using Fiji (Fiji is just ImageJ, version 1.51g).

Statistical analyses

Statistical analyses were performed using R Project software version 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria)³. Regarding baseline characteristics, continuous variables were presented as median with interquartile range (IQR) and categorical variables as number (n) with percentages. Cytokines mean signal intensity per μm^2 were compared using the paired t-test for comparison of infiltrated and non-infiltrated ROIs from patients, and unpaired t-test for comparison of ROIs from patients and HCs. P-values lower than 0.05 were considered statistically significant.

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Supplemental Table S1. Clinical and ophthalmological characteristics

A. Clinical characteristics						
Patient	Age[†]	Sex	History of conjunctivitis	EASI baseline	Time to onset of conjunctivitis (days)	
1	20	Male	No	25.6	56	
2	32	Female	Yes	16.4	112	
3	32	Male	Yes	16.8	56	
4	61	Female	Yes	34.8	38	
5	45	Male	No	12.3	63	
6	55	Male	No	24.1	70	
HC 1	72	Female	na	na	na	
HC 2	63	Male	na	na	na	

B. Ophthalmological characteristics						
Patient	Bulbar conjunctivitis	Palpebral conjunctivitis	Blepharitis	Limbitis	Cornea punctata	GC density (cells/mm)
1	ODS: mild	ODS: moderate	ODS: mild	No	No	5.9
2	No	ODS: moderate	No	No	No	1.4
3	ODS: severe	ODS: severe	ODS: moderate	ODS: moderate	ODS: moderate	3.3
4	No	No	ODS: mild	No	ODS: mild	3.3
5	OD: Mild	OD: Moderate OS: mild	OD: moderate OS: mild	OD: moderate	No	0
6	ODS: mild	ODS: moderate	ODS: moderate	No	No	1.9
HC 1	na	na	na	na	na	4.1
HC 2	na	na	na	na	na	9.8

[†] Age at initiation of dupilumab treatment. Ophthalmological characteristics were examined and graded in terms of severity by an experienced ophthalmologist following the Utrecht Ophthalmic Inflammatory and Allergic disease ocular surface score.¹ EASI, Eczema Area Severity Index.

Supplemental Table S2. Imaging Mass Cytometry antibodies

Antigen/Target	Clone	Metal tag	Dilution	Supplier	Catalogue number
E-cadherin	24E10	142 Nd	100	Cell Signaling Technology	CST3195BF
CD68	KP1	143 Nd	600	Thermo Fisher Scientific	14-0688-82
CD14	EPR3653	144 Nd	400	Abcam	ab214438
pS6	D57.2.2E	145 Nd	100	Cell Signaling Technology	CST4858BF
CD20	H1	146 Nd	150	BD Biosciences	555677
CD45RA	HI100	147 Sm	100	BioLegend	304102
HLA-DR+DP+DQ	CR3/43	148 Nd	200	Abcam	ab7856
IFN γ	D3H2	151 Eu	100	Cell Signaling Technology	CST8455BF
CD45RO	UCHL1	152 Sm	100	Cell Signaling Technology	CST55618BF
CD103	EPR4166(2)	153 Eu	200	Abcam	ab221210
TNF α	7B8A11	154 Sm	50	Proteintech	60291-1-Ig
Foxp3	236A/E7	155 Gd	50	Abcam	ab96048
CD4	EPR6855	156 Gd	50	Abcam	ab181724
T-bet	D6N8B	158 Gd	50	Cell Signaling Technology	CST13232BF
CD45	D9M8I	159 Tb	100	Cell Signaling Technology	CST13917BF
IL-10	polyclonal	160 Gd	150	R&D Systems	AF-217-NA
CD8 α	C8/144B	162 Dy	200	Thermo Fisher Scientific	14-0085-82
ICOS	D1K2T	163 Dy	100	Cell Signaling Technology	CST89601BF
PD-1	EPR4877(2)	165 Ho	50	Abcam	ab186928
CD56	NCAM1/784	166 Er	200	Abcam	ab216010
IL-17A	polyclonal	167 Er	100	R&D Systems	AF-317-NA
Ki-67	B56	168 Er	200	BD Biosciences	556003
GzmB	D6E9W	169 Tm	100	Cell Signaling Technology	CST46890BF
CD3	polyclonal	170 Er	100	Dako	A045229-2
CD11c	EP1347Y	171 Yb	100	Abcam	ab216655
CD69	polyclonal	173 Yb	150	Proteintech	10803-1-AP
TCR δ	H-41	174 Yb	100	Santa Cruz Biotechnology	sc-100289X
His H3	D1H2	176 Yb	600	Cell Signaling Technology	CST4499BF

Supplemental Figure S1

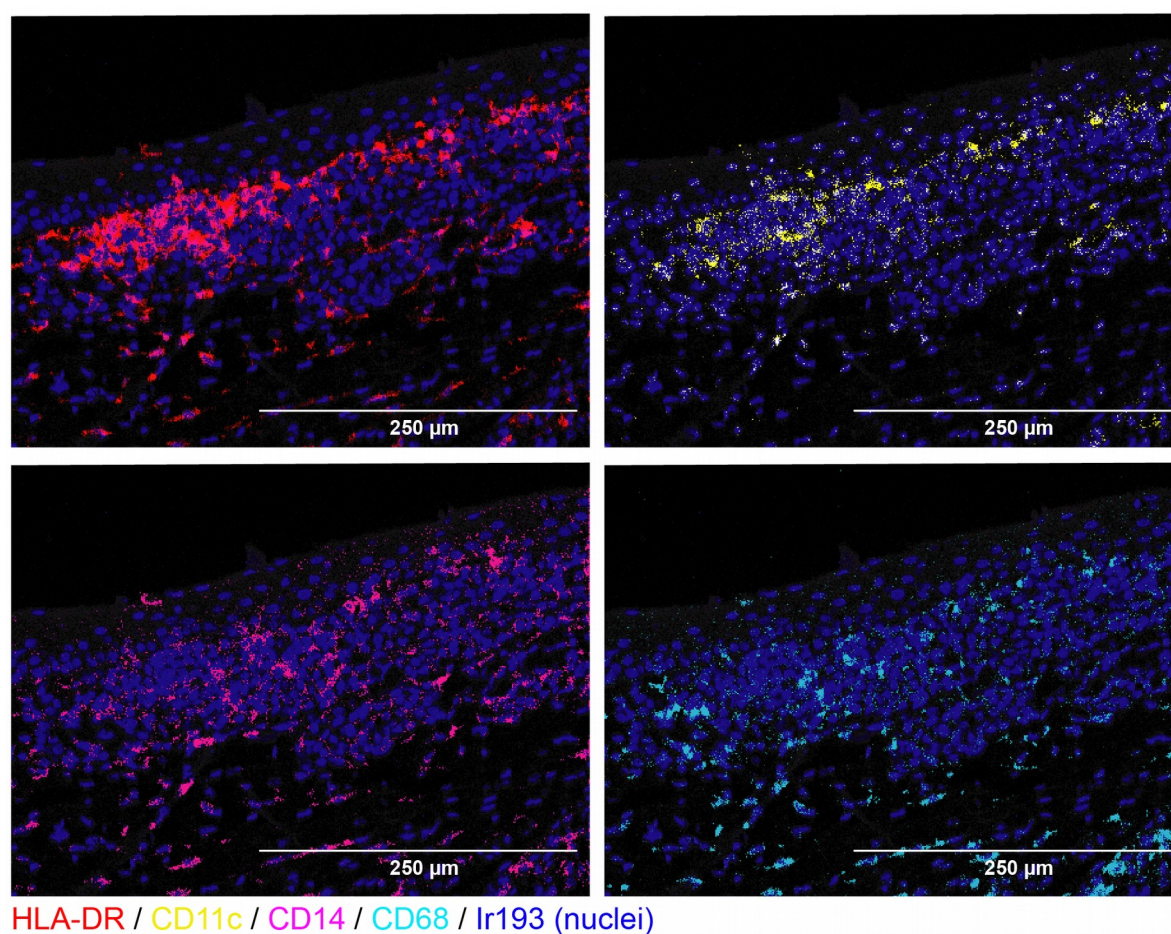
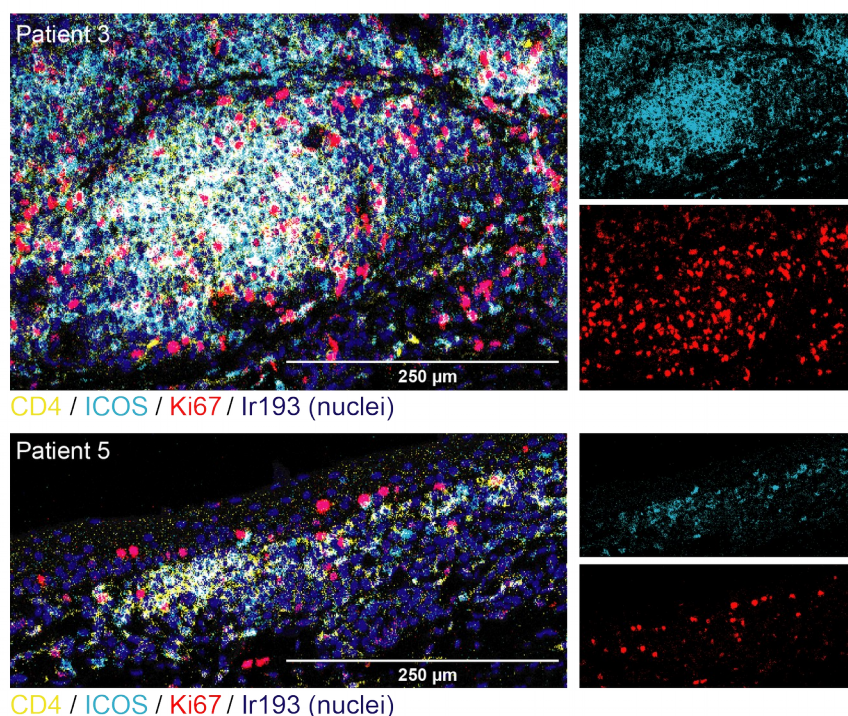


Figure S1. Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from AD patients developing conjunctivitis during dupilumab treatment. Representative image of patient 5 showing overlay of HLA-DR (red, upper left), CD11c (yellow, upper right), CD14 (magenta, lower left), and CD68 (cyan, lower right) with DNA intercalator (Ir) 193 (blue).

Supplemental Figure S2

A



B

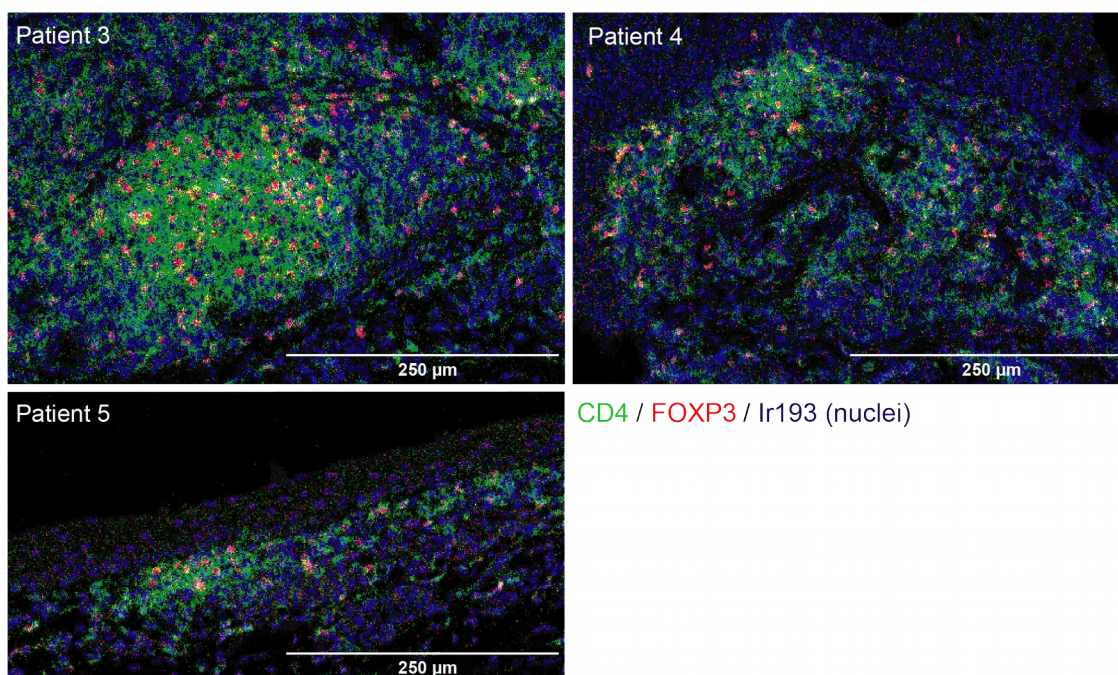


Figure S1. Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from AD patients developing conjunctivitis during dupilumab treatment. Representative image of patient 3 (A) showing overlay of CD4 (yellow), CD27/Inducible T-cell COStimulator (ICOS) (cyan), Ki67 (red), and intercalator (Ir) 193 (blue). Representative image of patient 3, 4 and 5 (B) showing overlay of CD4 (green), FOXP3 (red), and Ir 193 (blue), showing numerous regulatory T-cells within subepithelial infiltrates.

Supplemental Figure S3

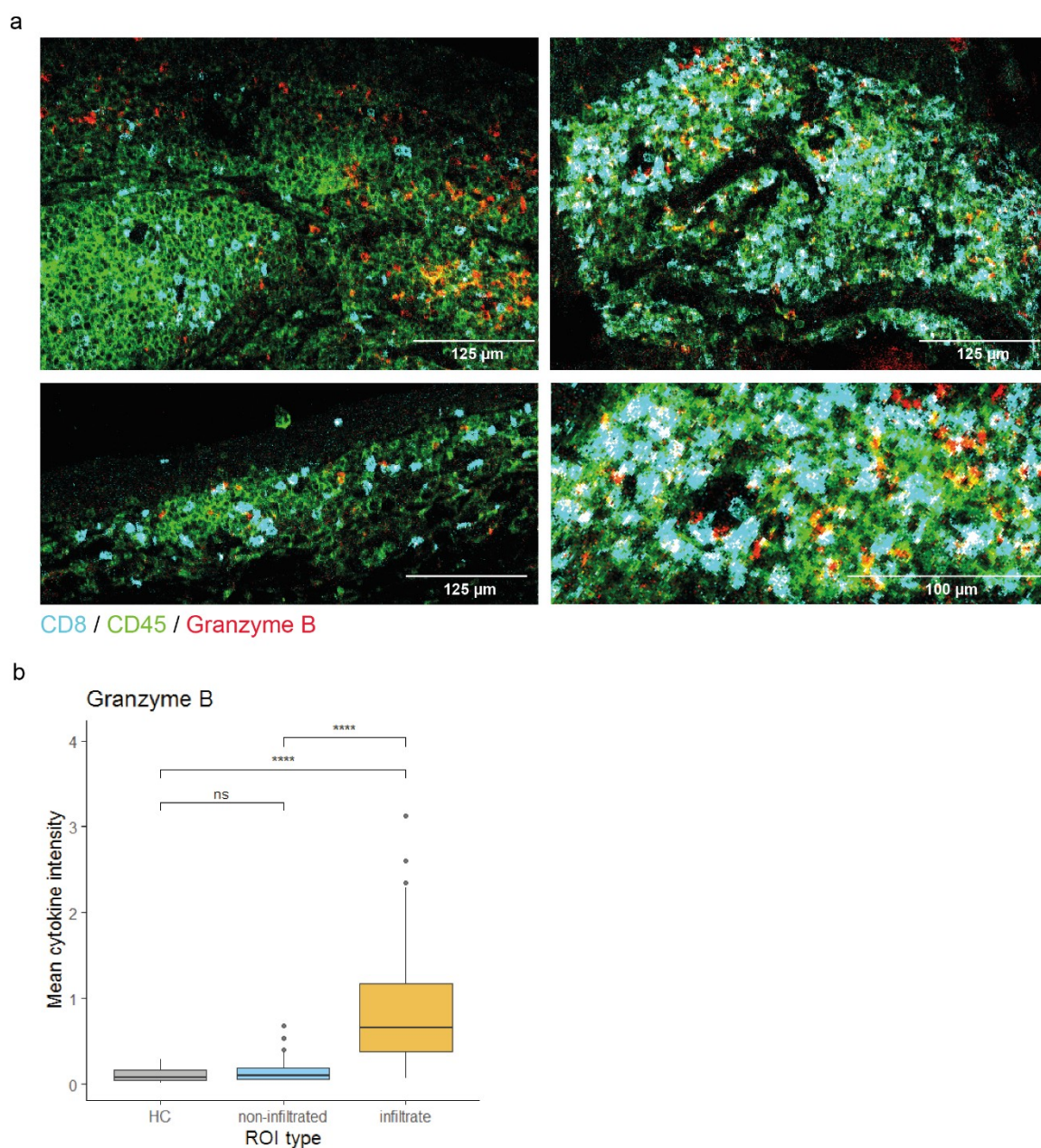


Figure S3. (A) Composite images derived from Imaging Mass Cytometry of conjunctival biopsy samples from patient 3 (upper left), patient 4 (upper and lower right), and patient 5 (lower left), showing overlay of CD8 (cyan), CD45 (green), and granzyme B (red). Magnified image of patient 3 (lower right) shows multiple CD8⁺ T-cells colocalized granzyme B, represented by white colored areas. **(B)** Mean cytokine signal intensity plotted granzyme B. Boxes represent medians with first and third quartiles (lower and upper hinges). The upper and lower whiskers extend from the hinge to the largest and smallest value, respectively, no further than 1.5* interquartile range. P-values lower than 0.05 were considered statistically

significant. Significance levels correspond to the following P values: *P < .05, **P < .01 and ***P < .005.

Supplemental Figure S4.

Figure S4. Representative histological images (first two rows) of haematoxylin and eosin (HE) and Alcian Blue staining, and Imaging Mass Cytometry (IMC) images of conjunctival biopsy samples from six AD patients developing conjunctivitis during dupilumab treatment, and two healthy control samples. IMC derived images of 27 antibodies, intercalator (Ir) 191 and 193 are displayed separately for all samples.

