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# Intravital imaging of the human cornea reveals the differential effects of season on innate and adaptive immune cell morphodynamics

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**Running head:** Seasonal effects on human corneal immune cell subsets

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**Key words:** Cornea, T cell, dendritic cell, macrophage, confocal

**Abbreviations:** DCs (dendritic cells), dSEARCH (dendrite surveillance extension and retraction cycling habitude), Fun-IVCM (Functional In Vivo Confocal Microscopy), NITBUT (non-invasive tear break-up time), OSDI (Ocular Surface Disease Index).

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**ABSTRACT**

**Purpose:** While the external environment has been shown to shape the systemic human immune landscape, defining the *in vivo* immune status of peripheral tissues has remained a technical challenge. We recently developed functional *in vivo* confocal microscopy (Fun-IVCM) for dynamic, longitudinal imaging of corneal immune cells in living humans. This study investigated the effect of seasonal-driven environmental factors on the density, morphology and dynamic behavior of human corneal immune cell subsets.

**Design:** Longitudinal, observational clinical study.

**Participants:** Sixteen healthy participants (18-40 years) attended two visits in distinct seasons in Melbourne, Australia (Visit 1: Spring/Summer: November-December 2021; Visit 2: Autumn/Winter: April-June 2022).

**Methods:** Environmental data were collected over each period. Participants underwent ocular surface examinations and corneal Fun-IVCM (Heidelberg HRT-3, Rostock Corneal Module). Volume scans (80µm) were acquired at 5.5±1.5 minute intervals, for up to five timepoints. Time-lapse videos were created to analyze corneal immune cells, comprising epithelial T cells and dendritic cells (DCs), and stromal macrophages. Tear cytokines were analyzed using multiplex bead-based immunoassay.

**Main Outcome Measures:** Difference in the density, morphological and dynamic parameters of corneal immune cell subsets over the study periods.

**Results:** Visit 1 was characterized by higher temperature, lower humidity, and higher air particulate and pollen levels than Visit 2. Clinical ocular surface parameters, and the density of immune cell subsets were similar across visits. At Visit 1 (Spring/Summer), corneal epithelial DCs were larger and more elongated, with a lower dendrite probing speed (0.38±0.21 vs 0.68±0.33µm/min, $p<0.001$) relative to Visit 2; stromal macrophages were more circular and had less dynamic activity (Visit 1: 7.2±1.9 vs Visit 2: 10.3±3.7 ‘dancing

index', $p<0.001$). T cell morphology and dynamics were unchanged across periods. Basal tear levels of IL-2 and CXCL10 were lower during Spring/Summer.

**Conclusion:** This novel study shows that the *in vivo* morphodynamics of innate corneal immune cells (DCs, macrophages) are modified by environmental factors, but such effects are not evident for adaptive immune cells (T cells). The cornea is a potential non-invasive, *in vivo* 'window' to season-dependent changes to the human immune system, with capacity to yield new insight into environmental influences on immune regulation.

## Introduction

In addition to the influence of intrinsic factors, such as age, sex and genetics, the human immune system is shaped by the external environment, including exposure to pathogens and other immune triggers. It follows that large variations in immune cell parameters have been described, even within healthy populations.[1] Immune heterogeneity is considered to be predominantly driven by non-heritable influences.[2] Some environmental effects are long-term, such as those induced by infections that lead to immunological memory.[3] Short-term exposures, such as seasonal factors, can also contribute to variations in immune cell characteristics.[4]

The effect of seasonal variations on the human immune system has been evaluated for several immune-related characteristics in circulating blood.[4-6] Higher neutrophil levels and a higher overall white blood cell count was reported in a US clinical cohort during Winter-Spring compared to Summer-Autumn.[7] Similarly, in a cohort of Western European origin, seasonal variations were noted in blood CD4+ and CD8+ T cell subsets, and cytokine production capacity after stimulation.[8] However, findings to date have been essentially limited to the evaluation of immune cell counts in the circulation, or *in vitro* cytokine responses. Potential seasonal-driven variations to local resident immune cells, in particular *in vivo* evaluations, have not yet been investigated in humans due to the inherent challenges of achieving intravital imaging of single cells in tissues.

The cornea is an ideal tissue to study features of the human mucosal immune system, as cell morphologies and dynamics can be directly captured, non-invasively in living humans using functional *in vivo* confocal microscopy (Fun-IVCM).[9] To date, studies of human corneal immune cells have been largely limited to the evaluation of static (single) images with quantification of parameters such as immune cell density and shape. Differences in corneal immune cell abundance and morphology have been reported in a spectrum of ocular and systemic diseases, relative to healthy controls, including dry eye disease,[10] allergy,[11] diabetes mellitus[12] and central nervous system degenerative disease.[13] However, the

influence of seasonal variations on corneal immune status has not yet been comprehensively studied.

Recent work by our laboratory has both successfully captured the dynamics of corneal subsets in living humans, and redefined understanding of the human corneal immune cell compartment by demonstrating that T cells constitutively patrol the healthy adult human corneal epithelium.[9] Using Fun-IVCM, we captured and quantified the *in vivo* dynamics of discrete immune cell subsets in human corneas, including intraepithelial DCs and T cells, and stromal macrophages, and evaluated their density, morphology, and dynamic behaviors in response to exogenous stimuli.[9] In the present study, we sought to investigate the influence of season-associated environmental factors on the density, morphology and dynamics of distinct corneal immune cell subsets in healthy individuals, and associated changes to inflammatory cytokine profiles in the tear fluid bathing the tissue.

## Materials and Methods

This study was conducted in accordance with the tenets of the Declaration of Helsinki, and approved by the University of Melbourne Human Research Ethics Committee (HREC ID #22828). Written informed consent to participate was obtained from all participants.

### *Participants*

The study involved 16 healthy participants (10 females, 6 males) in good general health, aged 18 to 40 years inclusive. All participants attended two visits that occurred about six months apart, over different seasons. Visit 1 (Spring/Summer) was in November or December 2021, aligning with the peak grass-pollen season in Melbourne, Victoria, Australia. Visit 2 (Autumn/Winter) was conducted in April to June 2022, coinciding with the lowest grass-pollen levels (Figure 1A). All visits occurred between 09.30 AM and 02.30 PM.

Participant inclusion criteria included an absence of current or recent ocular infection or injury, no history of eye surgery, no previous contact lens wear, an absence of any eye or systemic condition(s) that may affect ocular surface health, and not having had a COVID-19 or influenza vaccine within one week of both study visits. In addition, participants were required to have had no history of asthma, sinusitis, dust mite allergy, autoimmune or atopic disease.

*Environmental data collection*

Daily environmental data were collected over each study period for Visits 1 and 2. These data included: (i) hourly data for temperature (°C), relative humidity (%) and daily rainfall (mm) from the Australian Bureau of Meteorology Melbourne (Olympic Park) weather observation station; and (ii) $PM_{2.5}$ and $PM_{10}$ (μg/m³) air pollutant concentrations from the Alphington (Victoria, Australia) air monitoring site, obtained from the Environment Protection Authority Victoria. Daily grass concentrations were measured using a Burkard volumetric trap (Burkard Manufacturing Co. Ltd., Rickmansworth, Hertfordshire, UK) as previously described.[14] The Melbourne sampling site was located at the University of Melbourne, 1.7 km north of central Melbourne at coordinates −37.7971 and 144.9648. Samples were collected at 09:00 (local time) with the measured concentration representing the 24-hour average concentration from 09:00 the previous day in pollen grains/$m^3$ of air. The measured concentration is associated with the previous day the measurement was made, as it covers 62.5% of the measurement period. Daily grass pollen concentrations were categorized into the following levels: 'low' for an average concentration between 0 and 19 grains/$m^3$, 'moderate' for 20 to 49 grains/$m^3$, 'high' for 50 to 99 grains/$m^3$, and 'extreme' for concentrations of 100 grains/$m^3$ or more.[15]

*Ocular surface examination and Fun-IVCM imaging*

At each study visit, non-stimulated (basal) tear samples (~10 μL) were collected from the right eye using a glass microcapillary tube, and stored at -80°C until required for analysis. All

participants completed the Ocular Surface Disease Index (OSDI) questionnaire to evaluate for dry eye symptoms (score range, 0 to 100; score <13 no symptoms, 13-22 mild, 23-32 moderate, ⩾33 severe). To evaluate the frequency of eye rubbing, participants were asked to report how frequently they recalled rubbing their eyes over the past week (score range: never=0, rarely=1, sometimes=2, often=3, always=4). Participants underwent bilateral ocular surface clinical examinations including tear osmolarity (TearLab, Tear Corporation, USA), non-invasive tear break-up time (NITBUT) (OCULUS Keratograph K5M, OCULUS, Germany), corneal fluorescein staining and conjunctival lissamine green staining (quantified using the Oxford scale,[16] from 0 to 5. Conjunctival staining score was derived as the average from the temporal and nasal conjunctival areas). Ocular bulbar redness (averaged from the temporal and nasal regions), limbal redness (averaged from the temporal and nasal regions), eyelid redness (upper eyelid) and eyelid roughness (upper eyelid) were evaluated using the Brien Holden Vision Institute grading scale (range 0 to 4). Lid wiper epitheliopathy was graded using the Korb scale[17] (score range: 0 to 3, averaged for the upper and lower eyelids). Data from right eyes were used for all analyses, to match the eye that underwent corneal Fun-IVCM imaging.

Participants' right corneas were imaged using Fun-IVCM (Heidelberg Retina Tomograph-3 with Rostock Corneal Module, Heidelberg Engineering, Germany), per our published protocol.[9] In brief, the eye to be imaged received one drop of topical anesthetic (Oxybuprocaine hydrochloride 0.4%, Bausch & Lomb, Canada) prior to imaging. Repeated volume (*z*-stack) scans [400(x) × 400(y) × 80(z) μm] spanning the basal epithelium to mid-stroma were captured over a time interval up to 35 mins, at the corneal whorl (inferonasal paracentral cornea) for the latter half of enrolled participants (n=8). In addition, sequence scans, comprising 100 consecutive images acquired at the same corneal depth, were captured at the level of basal epithelium for both the corneal central and whorl regions, to quantify immune cell density and sensory nerve parameters (Figure 1B).

*Time-lapse video curation and analyses*

Fun-IVCM time-lapsed, immune cell videos were created using ImageJ software by generating stacks that combined four to five time-separate, unique images of the same corneal region, at a consistent tissue depth. For the epithelium, nerve axons were used as static landmarks to register the image set using the TrakEM2 plugin. For the stroma, time-lapse videos were created just below the anterior limiting lamina (corneal depth of 50 µm) (Figure 1B) using the Linear Stack Alignment with SIFT plugin.

Researchers masked to the study visit performed the immune cell analyses, using a suite of parameters to define the morphology and dynamic behaviors of each cell subset. Immune cell subsets were identified according to their distinct morphology and dynamic behaviors, as described previously.[9] Intraepithelial immune cells with multiple long dendrites were considered dendritic cells (DCs); smaller, motile cells without dendrites were classified as T cells. Macrophages in the stroma were identified in the time-lapse videos by their crawling behavior, distinct from static keratocytes, which showed minimal displacement of the hyper-reflective nucleus. For intraepithelial immune cells, eight to 12 non-overlapping static IVCM images were randomly selected from the acquired sequence scan sets to quantify cell density and morphology.[18] The evaluated cell morphological parameters included field area, cell area, perimeter, circularity, solidity and dendritic complexity (Table 1). Stromal macrophage density and morphology were evaluated from the time-lapse Fun-IVCM videos.

Dynamic cell behaviors were evaluated for parameters relevant to the immune cell subtype (Table 1). For DCs, dendrite dynamics were quantified using the dendrite tip probing speed and dendrite surveillance extension and retraction cycling habitude (dSEARCH).[19] The dSEARCH index is the cumulative distance of dendrite extension and retraction observed for a given DC, per unit of time. Dendrite tip probing speed was defined as the averaged distances of dendrite extension and retraction from all dendrite tips for a given DC, per unit of time. For macrophages, the ‘dancing index’ was used to evaluate the extent to which a cell’s shape changed between two timepoints.[20] After aligning the centroids of the

macrophages at each timepoint, the 'dancing index' was measured by their non-overlapped area divided by the total area, normalized to time. Data for immune cell densities, T cell motility parameters, DC dSEARCH index, and macrophage morphology at Visit 1 only have been reported previously.[9]

Corneal nerve parameters in the central and whorl regions of the cornea were quantified using the automated ACCMetrics software,[21] using the same IVCM image sets as for the intraepithelial immune cell density quantifications. The quantified nerve parameters were: corneal nerve fiber density (CNFD, density of total nerve fibers, fibers/mm$^2$), corneal nerve branch density (CNBD, density of the main nerve fiber branches, branches/mm$^2$), corneal nerve fiber length (CNFL, length of the nerve fibers, mm/mm$^2$), and corneal nerve total branch density (CTBD, density of total nerve fiber branches, total branches/mm$^2$).

*Tear cytokine analysis*

To confirm that the tear samples collected did not represent reflex tears, total tear protein concentration (µg/mL) and secretory IgA levels (ng/mL) in tear samples were assessed using NanoDrop (ThermoFisher, Waltham, MA, USA) and an in-house ELISA assay respectively, as previously described.[22] Cytokine levels were analyzed using bead-based multiplex immunoassay (Immune Monitoring 65-Plex Human ProcartaPlex Panel, ThermoFisher, Waltham, MA, USA) and performed according to the manufacturer's instructions using 3µl of tear sample diluted in 22µl of assay buffer. Samples were processed and acquired in the same analytical run on the FlexMAP 3D platform (Luminex). Concentration data for each cytokine were interpolated using ThermoFisher ProcartaPlex software.

*Statistical analysis*

Statistical analyses were performed using R software (Version 4.3.0, R Development Core Team, https://www.r-project.org/). Immune cell morphological and dynamic parameters were analyzed at the "per cell" level. A linear mixed-effects model was fitted to test the effect of

season, using a restricted maximum likelihood methodology. A random effect was included to account for the non-independence between observations from the same participant. For non-parametric data, Wilcoxon signed-rank test was performed. Data are shown as mean ± standard deviation (SD), unless otherwise indicated. A p-value less than 0.05 defined statistical significance.

## Results

*Environmental data*

Meteorological data in Melbourne, Victoria, Australia across each of the study visits are summarized in Table 2. Visit 1 (Spring/Summer) had higher temperatures ($p<0.001$ for both maximum and minimum temperatures), lower humidity ($p<0.001$), higher 10μm particulate matter concentrations ($PM_{10}$; $p=0.012$) and higher concentrations of grass pollen ($p<0.001$) relative to Visit 2 (Autumn/Winter). Notably, 2.5μm particulate matter concentrations were not statistically significantly different during the two visits. The average atmospheric grass pollen concentration was categorized as 'high' for Visit 1 and 'low' for Visit 2.

*Clinical characteristics and corneal nerve features of study participants*

All participants attended both study visits. Demographic and ocular characteristics are summarized in Table 3. Similar findings were observed for clinical ocular surface signs across the visits. Corneal nerve characteristics are summarized in Table 3. There were no changes to corneal nerve parameters between study visits.

*Corneal intraepithelial DCs show season-dependent differences in morphodynamics*

For corneal epithelial DCs (Figure 2A), cell density was similar across study visits in both the central and whorl regions (Figure 2B). The morphology of DCs was affected by season, with DCs generally having a larger, more elongated and more concave shape, with greater dendritic complexity, during Visit 1 (Spring/Summer) relative to Visit 2 (Autumn/Winter) (Figure 2C-H).

DCs showed a lower dendrite tip probing speed at Visit 1 ($p<0.001$) relative to Visit 2, but similar normalized dSEARCH index (Figure 2I-K, supplementary video 1). The larger DC field area and higher dendritic complexity at Visit 1 may compensate for the lower dendrite tip probing speed to achieve similar local tissue surveillance.

*Corneal intraepithelial T cell morphology and behaviors are independent of seasonal factors*

There was no difference in corneal intraepithelial T cell density across study visits (Figure 3A-B). Overall, season had no effects on T cell morphology (Figure 3C-F) or motility (Figure 3G-J).

*Corneal stromal macrophages show season-dependent differences in morphodynamics*

Season had no effect on corneal stromal macrophage density (Figure 4A-B). At Visit 1, macrophages were more circular and convex in shape (Figure 4C-F), and had lower dynamic activity (quantified using the 'dancing index') relative to Visit 2 (Figure 4G-K, supplementary video 2).

*Tear levels of IL-2 and CXCL10 show season-dependent variations*

The concentration of IL-2 in basal tears was higher at Visit 2 (Autumn-Winter) relative to Visit 1 (Figure 5A). Tear levels of CXCL10 were also higher during Visit 2 (Figure 5B). No difference was observed between the study visits for all other tear cytokines (Supplementary File 3).

## Discussion

There is growing evidence that environmental changes linked to seasonal variation can influence human immune status, including higher levels of neutrophils and white blood cell counts, and lower cytokine responses during Winter compared to Summer.[7,8] However, defining changes to human immune cells has been largely limited to the peripheral blood and their functions as evaluated *in vitro*.[8,23,24] The present study applied our recently developed non-invasive, time-lapse, intravital imaging of the human cornea (Fun-IVCM) to evaluate the *in vivo* morphological and dynamic features of distinct immune cell subsets. Our study provides the first *in vivo* evidence for differential immune cell behaviors in a local tissue (cornea) during different seasons in healthy humans. Moreover, we show distinct effects on myeloid but not lymphoid cell subsets. Compared to the Autumn-Winter period, during Spring-Summer corneal intraepithelial DCs were larger and more elongated, with a lower dendrite probing speed, and stromal macrophages were more circular and had less dynamic activity. Corneal intraepithelial T cells were unaffected by season.

In general, environmental factors can have more substantial effects on innate immune traits than adaptive immune features in the peripheral blood.[3] This effect may be due to innate immune cells being the so-called ‘first line of defense’ and thus having responsivity to environmental perturbations. The present study has consistent findings, with seasonal factors predominantly affecting the morphology and dynamics of presumed myeloid-derived immune cells (i.e., DCs and macrophages), with no effect on corneal T cells. The observed

higher dendrite probing speed of DCs and more dynamic morphological changes of macrophages during Winter might be interpreted as a higher level of cellular functional activity (e.g., antigen surveillance and presentation).[19,20,25] The less active profile of corneal innate immune cells may contribute to the incidence of *Pseudomonas aeruginosa* detected from ocular surface and the higher prevalence of corneal infection during summer.[26,27] Although other factors such as pathogen infectivity can influence the seasonal pattern of infectious diseases,[28] the patrolling activity of innate immune cells plays an crucial role in maintaining a free of pathogens and inflammation microenvironment.[29] On the other hand, the increased immune cell activity during Winter can be a response to pathogen, evidenced by that some organisms have a longer persistence and stronger transmission in cold temperatures.[30] Future investigations are needed to explore the relationship between immune cell dynamics and potential health impacts.

Our results align with previous research that has shown a more profound pro-inflammatory transcriptomic profile in the human immune system during Winter, in addition to annual periodical gene expression variations that were inverted between Europe and Oceania.[31] An increased pro-inflammatory state during Winter is also supported by higher circulating levels of C-reactive protein and IL-6 during this season.[31] We observed higher levels of the pro-inflammatory cytokines IL-2 and CXCL10 in human tears during the Autumn/Winter period relative to Spring/Summer. Akin to the known diurnal rhythm that has been described in the systemic human immune system, whereby plasma pro-inflammatory cytokine levels peak in the early morning,[32] changes to the pro-inflammatory status of the ocular surface may be driven by environmental alterations. This phenomenon might relate to the known higher incidence of autoimmune diseases during Winter such as rheumatoid arthritis and anterior uveitis.[33,34] Taken together, these findings suggest that the cornea could serve as an ideal and accessible peripheral tissue to define potential environmental influences on human immune homeostasis *in vivo*.

A recent meta-analysis reported high heterogeneity with respect to corneal intraepithelial immune cell density, evaluated using static IVCM images, in healthy individuals.[35] Although seasonal factors were not evaluated in this study, the authors proposed that methodological factors might be a contributor to the variability. Another likely influence is that prior studies have not recognized the presence of two distinct immune cell types (i.e., DCs and T cells) in healthy human corneal epithelium, as our group recently identified,[9] instead mis-categorizing all corneal epithelial immune cells as DCs. Using Fun-IVCM to unambiguously distinguish between these immune cell subsets, we provide more robust methodology to evaluate how these cell populations might be altered both during homeostasis and disease. In the present study, we newly identify that corneal intraepithelial DCs show season-dependent morphodynamic changes, but such alterations are not evident for T cells. This distinction provides additional supportive evidence that these two immune cell subsets likely behave differently in the corneal epithelium.

Corneal macrophages are primarily bone marrow-derived, with their functions in corneal health and disease having been widely explored in animal studies.[36,37] However, *in vivo* investigations of human corneal macrophages have historically been limited by the inability to distinguish stromal immune cells from keratocytes using static IVCM images. With dynamic, Fun-IVCM imaging, corneal stromal macrophages are readily differentiated from stationary tissue features by their crawling behavior between keratocytes (Supplementary video 2). Corneal stromal macrophages were found to have a more circular and convex shape in Spring/Summer, and a more elongated morphology in Autumn/Winter. Macrophage shape could relate to cell polarization or activation state. Macrophages polarized towards a M1 state (pro-inflammatory) have a rounded shape, while M2 polarization leads to cellular elongation, with the cell area remaining constant.[38] Together, these findings suggest that seasonal factors may influence the phenotypic polarization of corneal stromal macrophages, to assume a more circular, pro-inflammatory M1 phenotype during Spring/Summer. The finding of a lower ‘dancing index’ (dynamic behavior) in Spring/Summer relative to

283 Autumn/Winter, may indicate that cell dynamics are distinct from polarization state. Future
284 investigations are warranted to explore the behavioral significance of the macrophage
285 'dancing index' *in vivo*.

286 Many factors may contribute to the observed seasonal variations in human corneal immune
287 status, including sun exposure (vitamin $D_3$), pollen concentration, temperature and
288 atmospheric particle concentration.[6,24,39,40] Although we are unable to determine which
289 specific factor(s) induced the observed seasonal differences in corneal immune cell
290 morphology and behavior, pollen concentration may not be the dominant factor. A recent
291 study evaluated (presumed) corneal DC morphological changes between asymptomatic
292 (Winter) and symptomatic phases (Summer) in individuals with allergic conjunctivitis.[41] The
293 authors reported less DCs with long dendrites during Winter in these pollen-sensitive
294 individuals, which is similar to our findings in healthy individuals. It is worth noting that the
295 environmental conditions in the weeks leading up to each visit were not factored into our
296 analysis, which may induce cumulative or lagged effects on immune cell behavior. Together,
297 these findings suggest that variations in corneal immune cell characteristics across different
298 seasons are likely multifactorial, involving a complex interplay of environmental and
299 biological factors.

300 The sample size (n=16 participants) for this longitudinal study should be considered when
301 interpreting the findings. To, at least in part, offset this factor, the analysis adopted a 'per cell'
302 approach, with appropriate statistical methods (mixed effect models) to account for within-
303 person correlation and differences in cell sampling between participants; this enables more
304 informative comparisons than analyzing averaged data at the participant level. In view of
305 this, the present study can be interpreted to provide 'proof of principle' that the dynamics of
306 resident immune cells in peripheral tissues (i.e., the cornea) are affected by seasonal-driven
307 environmental factors. Larger clinical studies would be beneficial to validate these
308 observations, including to expand the consideration of potential contributory factors; for
309 example, a multi-center study involving sites in the Southern and Northern hemispheres,

which evaluates potential additional contributing factors, such as sun exposure, habitual environments and microbial exposures.

In conclusion, this study uniquely describes the influence of season on immune cell behaviors *in situ* in a living human cohort. Season-dependent differences in cell morphodynamics were most apparent in innate immune cells (intraepithelial DCs and stromal macrophages). These findings identify the cornea as a valuable, intact tissue for investigating the effect of external environmental factors on the human immune system, enabling longitudinal quantification of the *in vivo* morphology and dynamic behavior of distinct immune cell subsets. In addition, this study highlights the importance of considering the influence of season in the design, data collection and interpretation of longitudinal clinical studies evaluating immune cells (in health and diseased populations), as this could otherwise act as potential confounding factor that affects the validity of the reported findings.

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## FIGURE LEGENDS

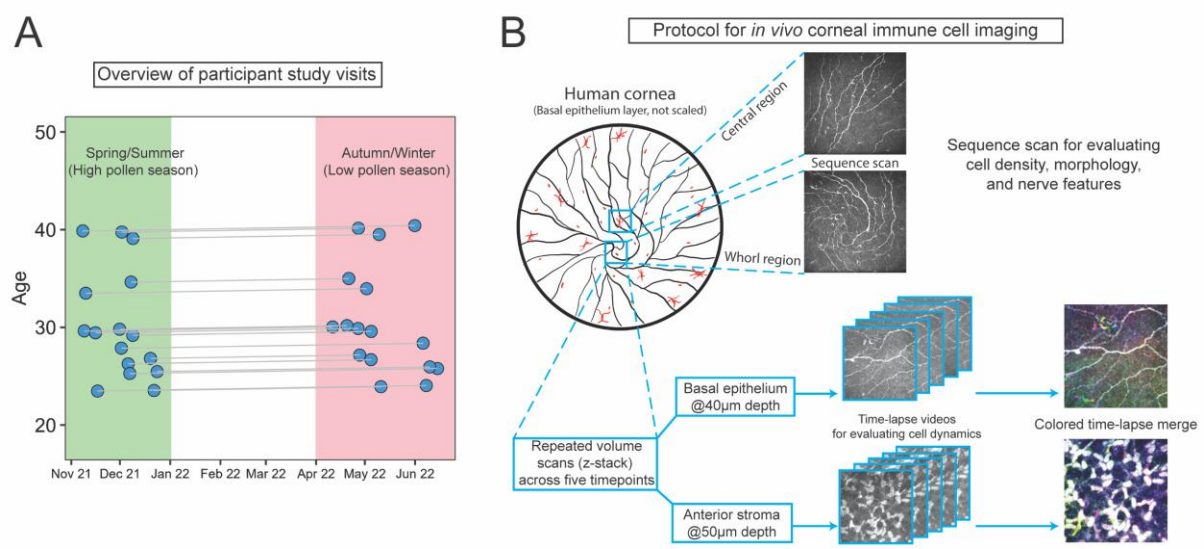



**Figure 1.** Summary of the study design and *in vivo* confocal microscopy imaging (IVCM) protocol. **(A)** Healthy adult participants aged between 20 and 40 years attended two visits. **(B)** Fun-IVCM was performed in the corneal whorl region, and static IVCM images were collected from both the whorl and central cornea.

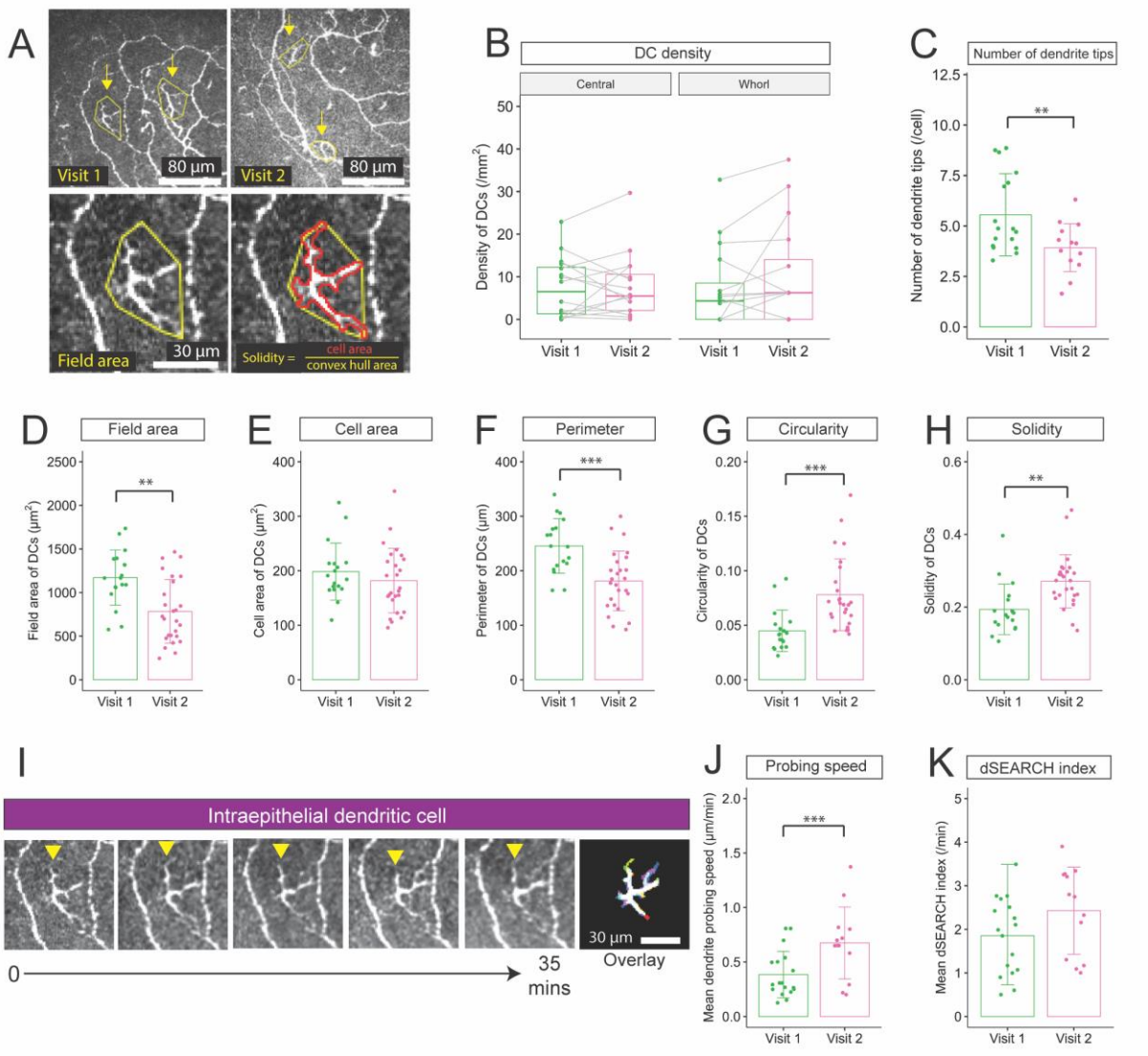



**Figure 2. Season-dependent changes to the morphodynamics of corneal intraepithelial DCs**. The morphology and behavior of DCs were significantly affected by season, with cells generally having a larger, more elongated and more concave shape, and a greater dendritic complexity, but a lower dendrite tip probing speed, during the Visit 1 (Spring/Summer) relative to Visit 2 (Autumn/Winter). **(A)** Representative corneal in vivo confocal microscopy images showing DCs in the basal epithelium, with their field area (yellow) and cell area (red) delineated, in a representative participant at Visits 1 and 2. **(B)** DC density was similar at each study visit. **(C-H)** Effect of season on various morphological features of DCs. At Visit 1, DCs had more tips (C), a greater field area (D), no change in cell area I, larger cell perimeter (F), lower circularity (G) and lower solidity (H). **(I)** Representative time-lapse Fun-IVCM images showing DC probing behavior. **(J-K)** Effect of season on the dynamic behavior of DCs. At Visit 1, DCs had a lower probing speed (J) but similar dSEARCH index (K) compared to Visit 2. **p<0.01, ***p<0.001. Data are shown at the 'per participant' level for dendritic cell density (Panel B), and 'per cell' level for morphology and dynamic parameters (Panels C-H, J-K), with the latter involving a mixed effects model for the statistical analysis.

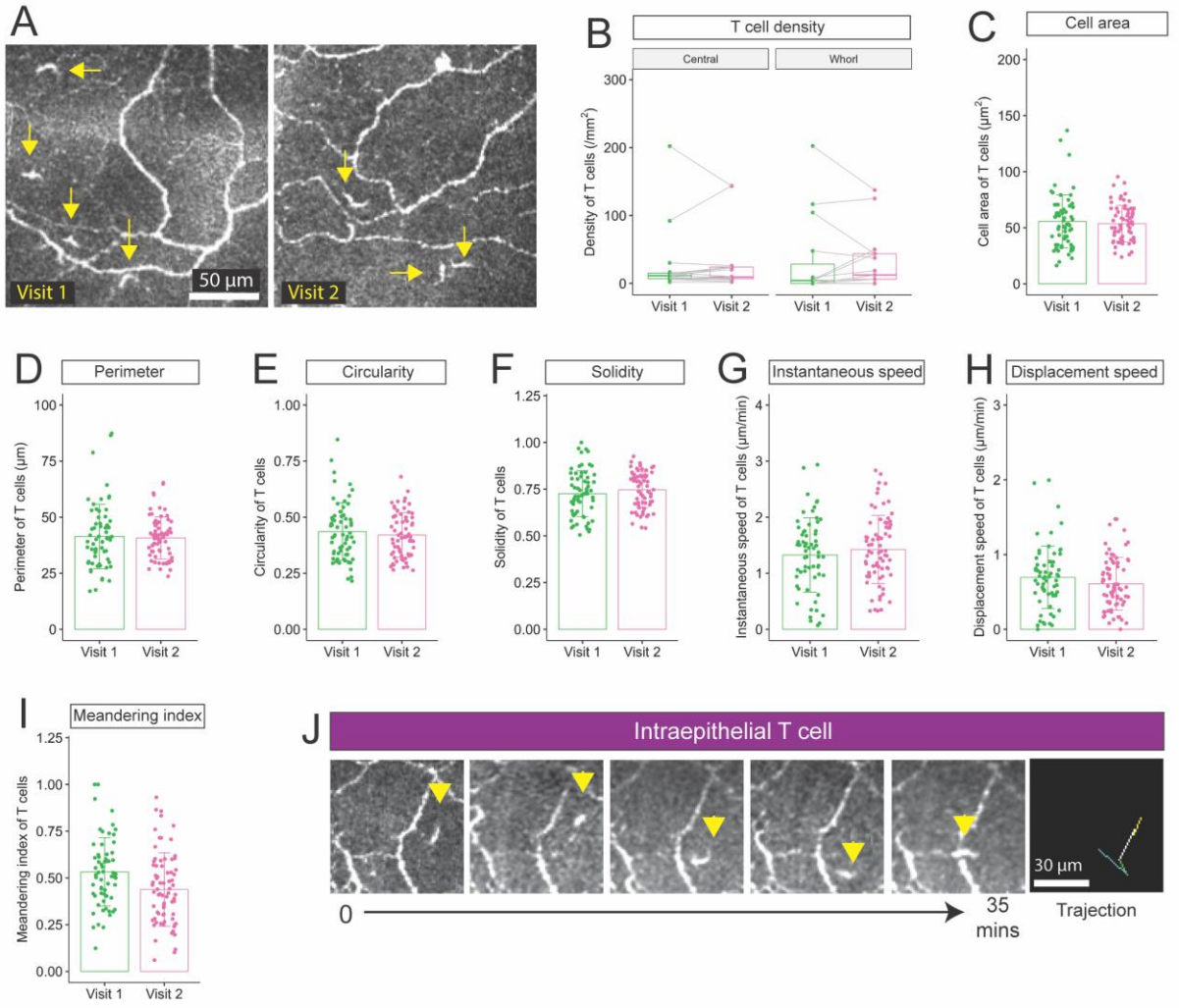
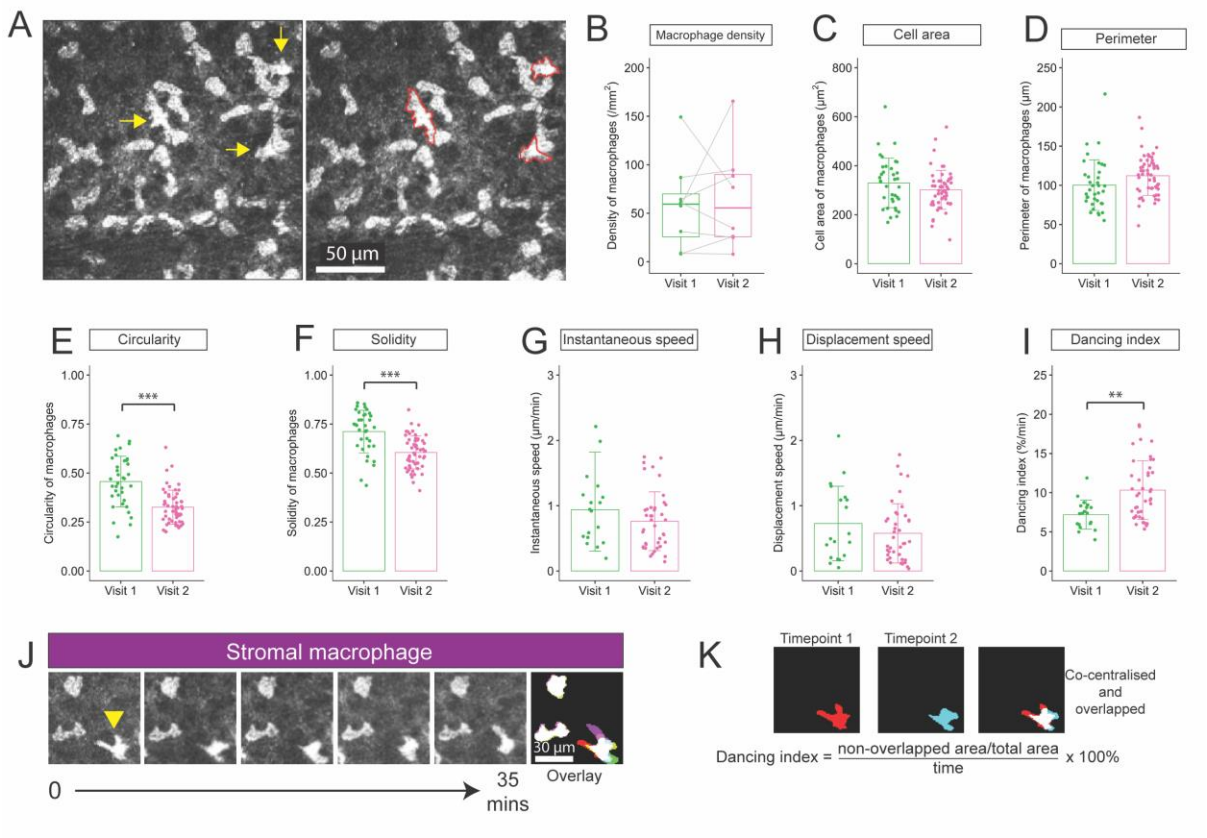



**Figure 3. The morphology and dynamics of corneal intraepithelial T cells are unaffected across seasons. (A)** Representative IVCM images showing T cells (arrows) at the level of corneal sub-basal nerve plexus. **(B)** Plot showing similar T cell densities in the central and whorl regions, at both study visits. There was an absence of season-dependent differences for T cell morphology, for each of cell area **(C)**, perimeter **(D)**, circularity **(E)** and solidity **(F)**. T cell dynamic behaviors were also unaffected, with no differences in instantaneous speed **(G)**, displacement speed **(H)** or meandering index **(I)** across seasons. **(J)** Representative time-lapse IVCM images showing the patrolling behavior of a T cell; the final panel shows the movement trajectory of the cell. Data are shown at the 'per participant' level for T cell density (Panel B), and 'per cell' level for morphology and dynamic parameters (Panels C-I), with the latter involving a mixed effects model for the statistical analysis.

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490

491 **Figure 4. Season-dependent changes to the morphodynamics of corneal stromal**
492 **macrophages.** The morphology and behavior of stromal macrophages were affected by
493 season, with cells having a more circular and convex shape, and lower dynamic activity,
494 during Visit 1 (Spring/Summer) relative to Visit 2 (Autumn/Winter). **(A)** Representative IVCM
495 images showing macrophages in the superficial corneal stroma (yellow arrows and red cell
496 outlines). These macrophages are challenging to differentiate from static keratocytes (white
497 cells without indicators) from static IVCM images but are readily distinguishable in the time-
498 lapse Fun-IVCM videos based on their movement. **(B)** Corneal stromal macrophage density
499 was unchanged across visits. There were no differences for cell area **(C)** or perimeter **(D)**
500 across seasons, but cell circularity **(E)** and solidity **(F)** were higher at Visit 1 relative to Visit
501 2. For dynamic cell behaviors, there was no inter-season difference for instantaneous speed
502 **(G)** or displacement speed **(H)**, but cells showed a lower 'dancing index' at Visit 1 relative to
503 Visit 2 **(I)**. **(J)** Representative images showing a stromal macrophage changing its
504 morphology over the imaging period of a Fun-IVCM time-lapse video. **(K)** Representative
505 images showing how the 'dancing index' is calculated for a single cell, to quantify the change
506 in macrophage shape over time. **p<0.01, ***p<0.001. Data are shown at the 'per
507 participant' level for stromal macrophage density (Panel B), and 'per cell' level for
508 morphology and dynamic parameters (Panels C-I), with the latter involving a mixed effects
509 model for the statistical analysis.

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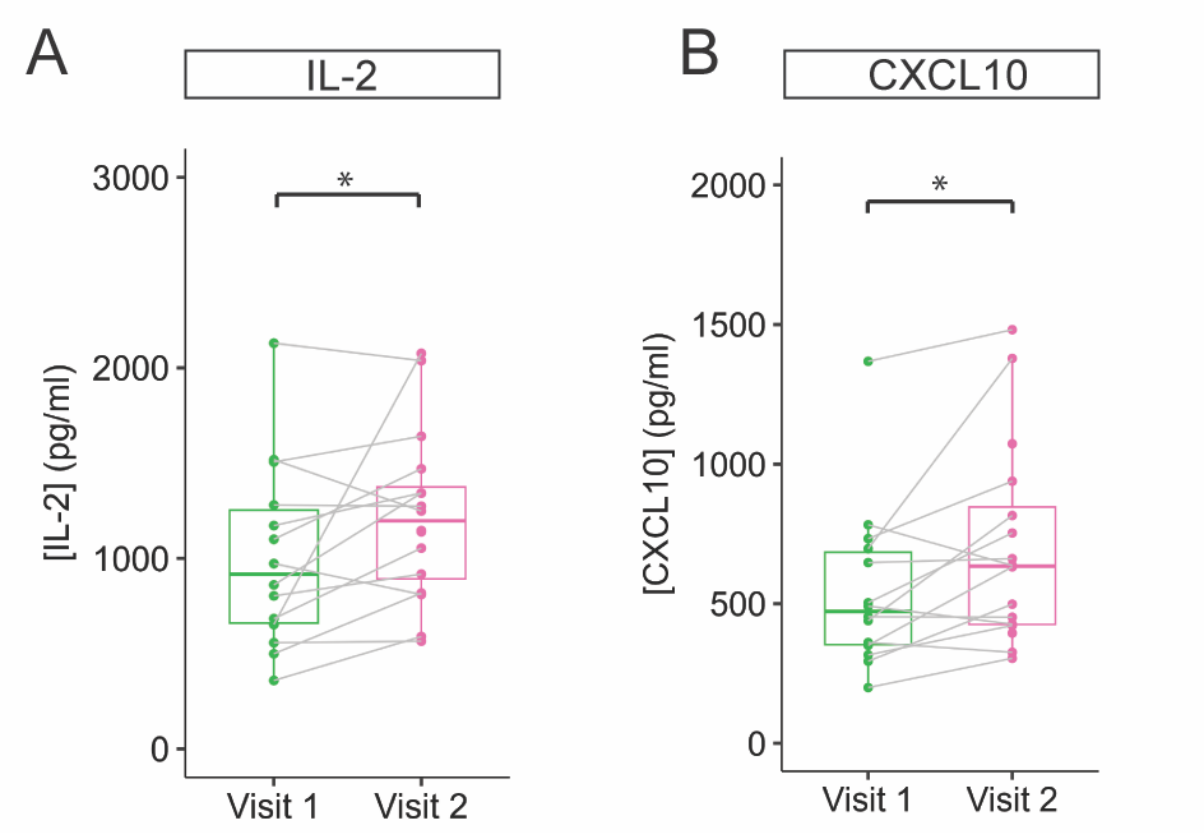


**Figure 5.** Tear cytokine differences across study visits. **(A)** Lower levels of tear IL-2 level were evidence Visit 1 compared to Visit 2. **(B)** Similar findings were observed for CXCL10, which were also relatively higher at Visit 2. *p<0.05.