In Vivo Confocal Microscopy Demonstrates Increased Immune Cell Densities in Corneal Graft Rejection Correlating With Signs and Symptoms

CHAREENUN CHIRAPAPAISAN, ALESSANDRO ABBOUDA, ARSIA JAMALI, RODRIGO T. MÜLLER, BERNARDO M. CAVALCANTI, CLARA COLON, DEBORAH WITKIN, AFSUN SAHIN, REZA DANA, ANDREA CRUZAT, AND PEDRAM HAMRAH

• PURPOSE: Diagnosis of graft rejection is based on patient symptoms and on clinical signs detected by slit-lamp biomicroscopy. This study investigated whether laser in vivo confocal microscopy (IVCM) can aid in the diagnosis of corneal graft rejection by detecting cellular corneal changes that take place after transplantation.

• DESIGN: Prospective case-control study.

• SUBJECTS: Thirty-eight eyes of 38 patients with penetrating keratoplasty (15 eyes with corneal graft rejection, 23 eyes without rejection) and 9 age-matched normal controls.

• METHODS: Laser IVCM was performed in the corneal grafts centrally. The density of immune cells (IC) was assessed for epithelial, sub-epithelial, stromal, and endothelial layers by 2 masked observers. IC density was compared among different groups and correlated to clinical signs and symptoms of corneal graft rejection.

• MAIN OUTCOME MEASUREMENTS: Outcome measurement was the IC density in the corneal layers and its associations with the presence of clinical signs and symptoms of corneal graft rejection.

• RESULTS: The IC density was significantly different between rejected and non-rejected grafts ($P = 0.004$) and different from that of normal controls ($P = 0.001$). Among corneal layers, IC density was significantly higher in rejected grafts than in non-rejected grafts in only the sub-basal (611.54 ± 573.74 vs. 340.61 ± 268.60 cells/mm^2, respectively; $P = 0.049$) and endothelial layers (250.62 ± 267.13 vs. 103.47 ± 81.91 cells/mm^2, respectively; $P = 0.001$). Patients with decreased best corrected visual acuity, Khodadoust line, and anterior chamber cells demonstrated a significant increase in total IC density ($P < 0.05$), whereas patients with symptoms of irritation, light sensitivity, and pain revealed a specific increase in IC density in the sub-basal layer ($P < 0.05$). Patients with ocular pain had higher IC density in the epithelial layer than those without pain ($P = 0.03$).

• CONCLUSIONS: Patients with corneal graft rejection demonstrate a significant increase in corneal immune cells, particularly, in the sub-basal and endothelial layers compared to patients with non-rejected grafts and controls. Although symptoms associated with endothelial rejection demonstrate a general increase in IC, pain, irritation, and light sensitivity are associated with increased IC in the sub-basal layer. Assessment of patients with corneal graft rejection by IVCM may serve as an adjunctive tool in the diagnosis and management of corneal graft rejection. (Am J Ophthalmol 2019;203:26–36.

Abbreviations: AC = anterior chamber; APC = antigen-presenting cell; AS-OCT = anterior segment optical coherence tomography; IC = immune cell; IVCM = in vivo confocal microscopy; KP = keratic precipitate; NV = neovascularization; PKP = penetrating keratoplasty

CORNEAL OPACITIES ARE A LEADING CAUSE OF VISUAL impairment and blindness worldwide.[1] Corneal transplantation, or keratoplasty, can restore corneal transparency and visual function. Approximately 100, 000 to 150, 000 corneal transplants are performed annually, not counting the transplantation technique.[2] The success rates of corneal transplants are considerably higher than other solid organ grafts, given that the cornea is an immune-privileged tissue.[3,4] However, favorable outcomes appear to be limited to patients who undergo "low-risk" keratoplasty, with survival rates of 90% and 82% at 5 and 10 years post-transplantation, respectively.[5–9] In contrast, rejection

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From the Ocular Surface Imaging Center (C.C., A.J., R.T.M., B.M.C., C.C., D.W., R.D., A.C., P.H.), Cornea and Refractive Surgery Service, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, USA; and Center for Translational Ocular Immunology, and Cornea Service (A.A., A.J., R.T.M., A.S., P.H.), New England Eye Center, Department of Ophthalmology, Tufts Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA.

Inquiries to Pedram Hamrah, Department of Ophthalmology, Tufts Medical Center, 800 Washington Street, Boston, Massachusetts 02111, USA; e-mails: phamrah@tuftsmedicalcenter.org; p_hamrah@yahoo.com

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rates after penetrating keratoplasty (PKP) into "high-risk" recipient beds can be as high as 70%.[5,10–15]

Generally, the major compromising factor in corneal graft failure is immunological rejection.[16] Although early graft rejection can be reversible, chronic rejection is irreversible and leads to progressive endothelial cell loss, subsequent corneal edema, and eventual graft failure. The incidence rates of immune-mediated graft rejection vary greatly between 2.3% and 68%,[17] depending on the patient's risk factors. Previous studies have reported that graft rejection has occurred in 40% to 70% of patients with high-risk grafts during the first postoperative year.[12–14] However, the recent evolution of new surgical techniques of lamellar and endothelial keratoplasties combined with improved graft preservation and postoperative medications have resulted in enhanced success rates. Nevertheless, allograft rejection cannot be completely eliminated and remains the greatest hurdle for long-term graft survival.

Pre-operative and post-operative ocular inflammation contribute to corneal neovascularization (NV) and formation of lymphatic vessels in the cornea. An increase in lymphatic vessels allows for enhanced egress of corneal antigen-presenting cells (APCs) to the draining lymph nodes, were they present donor antigens to T lymphocytes, generating allosensitization.[18] New blood vessels allow for enhanced influx of T effector cells and additional APCs into corneal grafts. Activated and sensitized T cells recognize corneal antigens and may trigger immune-mediated graft rejection. Consequently, the corneal graft becomes edematous, and tissue clarity is reduced. To inhibit this devastating immune process and to recover corneal transparency, early detection of the immune responses and timely treatment are essential. Typically, diagnosis of corneal allograft rejection is based on the patient's symptoms and clinical signs detected by slit-lamp biomicroscopy.[18] Unfortunately, early graft rejection may be missed on examination. Furthermore, several conditions, such as infections, may confound the signs and symptoms of immune rejection, resulting in missed or delayed diagnosis.[17] The use of specular microscopy or anterior segment optical coherence tomography (AS-OCT) have been introduced to detect early signs of corneal graft rejection.[19–21] Specular microscopy can only visualize the corneal endothelial layer, and AS-OCT can only demonstrate an increase of endothelial/Descemet's membrane complex thickness but not cellular changes in other corneal layers.[19–22] Thus, these techniques are not being routinely used in clinical practice to assess graft rejection.

In vivo confocal microscopy (IVCM) has been widely used in the examination of several corneal diseases.[23–28] The technology provides high-resolution images of the intact, living cornea at the cellular level.[29] This quasihistological view facilitates the investigation of microstructural changes and intracorneal immune cells, similar to histopathological methods, aiding in the diagnosis of obscure corneal conditions or processes that may be missed on routine slit-lamp examinations.[30] In the current study, using laser IVCM, we analyzed the immune cell (IC) density in corneal grafts in patients who had previously undergone PKP and compared the results between rejected and non-rejected grafts.

METHODS

• **STUDY PARTICIPANTS:** The Massachusetts Eye and Ear Infirmary (MEEI) Institutional Review Board/Ethics Committee approved this prospective case-control study. The study was carried out in compliance with the principles of the Declaration of Helsinki, the International Conference on Harmonisation, and the Good Clinical Practice recommendations. Informed consent was obtained from all subjects prior to the start of the study. Inclusion criteria for the non-rejected corneal transplantation control group were 18 years of age or older, a history of PKP between 4 and 8 weeks prior to enrollment, and a clear graft for at least 1 month post-operatively. These patients were re-examined at their 1-year follow-up appointment. For the graft rejection group, inclusion criteria were clinical diagnosis of graft rejection by a cornea specialist (P.H. or R.D.). Exclusion criteria included uncontrolled glaucoma, contact lens use within 3 months, any ocular surgeries after corneal transplantation, and recent infectious keratitis within 3 months. For the control PKP group, previous graft rejection was an additional exclusion criterion. The indications for corneal transplantation were optical or therapeutic, including pseudophakic bullous keratoplasty, Fuchs' endothelial corneal dystrophy, corneal scars, keratoconus, previous graft failure, and infectious keratitis with failed medical therapy. The standard postoperative topical corticosteroid regimen for the non-rejected PKP group included prednisolone acetate 1% eye drops taken 4 times daily for the first 3 months. This therapy was then gradually tapered to once daily, depending on underlying cause, level of inflammation, rejection episodes, level of corneal vascularization, and other relevant factors. Nine eyes from 9 subjects were recruited from healthy volunteers, and patients were routinely evaluated during clinic and served as normal control subjects.

In total, 38 eyes with a history of PKP from 38 patients were recruited. The PKP control group included 23 patients with clear non-rejected grafts. The graft rejection group included 15 eyes from 15 patients that had presented with corneal allograft rejection as diagnosed by a cornea specialist and in whom it was decided treatment for graft rejection would be initiated. These 15 patients had clear grafts for at least 1 month post-operatively prior to presentation with corneal graft rejection. The diagnostic criteria of corneal allograft rejection were based on clinical signs and symptoms, including decreased vision, ocular

irritation, light sensitivity, ocular pain or pressure, ciliary injection, progressive corneal NV, corneal edema, or presence of anterior chamber (AC) cells. Specific clinical signs also were considered, including an epithelial rejection line, subepithelial infiltration, keratic precipitates (KPs), or a Khodadoust line.[18] All patients with non-rejected grafts presented to the MEEI Cornea Service for regular follow-up examinations. Patients with graft rejection presented for scheduled appointments or urgent visits due to abnormal ocular symptoms. All patients and control subjects underwent slit-lamp biomicroscopy. Topical steroid therapy status at the time of examination for each patient group is shown in Supplemental Table 1.

• **IN VIVO CONFOCAL MICROSCOPY:** Laser IVCM (Retina Tomograph 3 with Rostock cornea module [HRT3/RCM], Heidelberg Engineering GmbH, Heidelberg, Germany) was performed unilaterally in the central cornea of each subject. Patients in the non-rejected control PKP group were imaged twice, once at baseline between 4 and 8 weeks after transplantation and then again at their 1-year follow-up examination. The purpose for the follow-up was to allow assessment both after the initial post-operative phase and after 1 year of stable transplantation without the use of high-dose topical corticosteroids. The microscope uses a 670-nm diode light source and is equipped with a 63× objective immersion lens with a numerical aperture of 0.9 (Olympus, Tokyo, Japan) to generate a 400- × 400-μm coronal image (384 pixels × 384 pixels) with 800-fold object magnification. Prior to initiating a corneal scan, an anesthetic eye drop (0.5% proparacaine hydrochloride eye drop; Alcon, Fort Worth, Texas) was first applied to both eyes of each subject, followed by a drop of lubricating gel (2.5% hydroxypropyl methylcellulose; GenTeal; Novartis Ophthalmics, East Hanover, New Jersey). A sterile, disposable poly(methylmethacrylate) cap (Tomo-Cap, Heidelberg Engineering) was filled with a layer of GenTeal and mounted on the HRT3-RCM optics. GenTeal gel was also applied to the outside tip of the Tomo-cap. The microscope was advanced manually until the gel from the cap contacted the gel placed on the corneal surface. Full-thickness corneal scans were performed centrally, using the instrument's sequence mode function. Multiple scans of 4 corneal layers (epithelium, sub-basal layer, anterior stroma, and endothelium) were collected, and images were selected for each layer. The sub-basal layer is immediately posterior to the basal epithelium and anterior to Bowman's layer. The anterior corneal stroma was defined as stromal tissue located 50 μm beneath the Bowman's layer. The endothelium was the most posterior corneal layer with a characteristic pattern of hexagonal cells with hyporeflective borders. Five representative well-focused, high-contrast confocal images of each layer were selected for analysis of IC density by a masked observer.

• **ANALYSIS OF CORNEAL IMMUNE CELL DENSITY:** Two masked observers (C.C., A.A.) manually analyzed the ICs in the basal epithelium, sub-basal layer, anterior stroma, and endothelium. The ICs were identified and quantified based on their shapes and sizes: corneal dendritiform cells (DCs) were characterized by their bright individual dendritiform structure, made up of the cellular body and dendrites (Figure 1). In the epithelial and sub-basal layers, DCs were predominant among the ICs. In contrast, stromal DCs were more variable in shape, with nuclei that were neither round nor ovoid, but bean-shaped.[31,32] These atypical hyper-reflective cells in the stromal layer were differentiated from activated keratocytes by manually adjusting image contrast (Figure 2). In the endothelial layer, ICs were identified as round, dense, hyper-reflective structures that exceeded 10 microns, which is the minimal size of human immune cells.[33] The IC densities were quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland). The primary IC count from each confocal image was converted from cells per frame area (0.16 mm^2) to cells per mm^2 before comparing the results among the groups.

• **STATISTICAL ANALYSIS:** Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL). Chi-square test was used to evaluate the differences among qualitative variables. Analysis of variance (ANOVA) with least-significant difference (LSD) post-hoc test was applied to assess the differences in the quantitative variables (age and density of inflammatory cells in each corneal layer) among the controls and patients with and without corneal graft rejection. Student's *t*-test was used to evaluate the differences in the quantitative variables between two groups, including the comparisons of baseline characteristics of two patient groups and assessment of the inflammatory cell density in each corneal layer based on the presence of clinical signs and symptoms of corneal graft rejection. Step-wise multiple linear regression analysis was performed to assess independent correlations between clinical signs and symptoms with IC densities with an entry level of $P = 0.10$. A P value less than 0.05 was considered statistically significant for each test.

RESULTS

THIRTY-EIGHT EYES OF 38 PATIENTS WHO HAD PREVIOUSLY undergone PKP (23 with non-rejected grafts and 15 with rejected grafts) were included in this study. Nine eyes from 9 healthy, age-matched volunteers served as controls. Demographic data of patients with PKP groups and normal control subjects are presented in Table 1. The post-operative time of rejection in patients with rejected grafts was 992.13 ± 81.26 days, respectively ($P < 0.001$). No significant differences were observed among the frequencies of

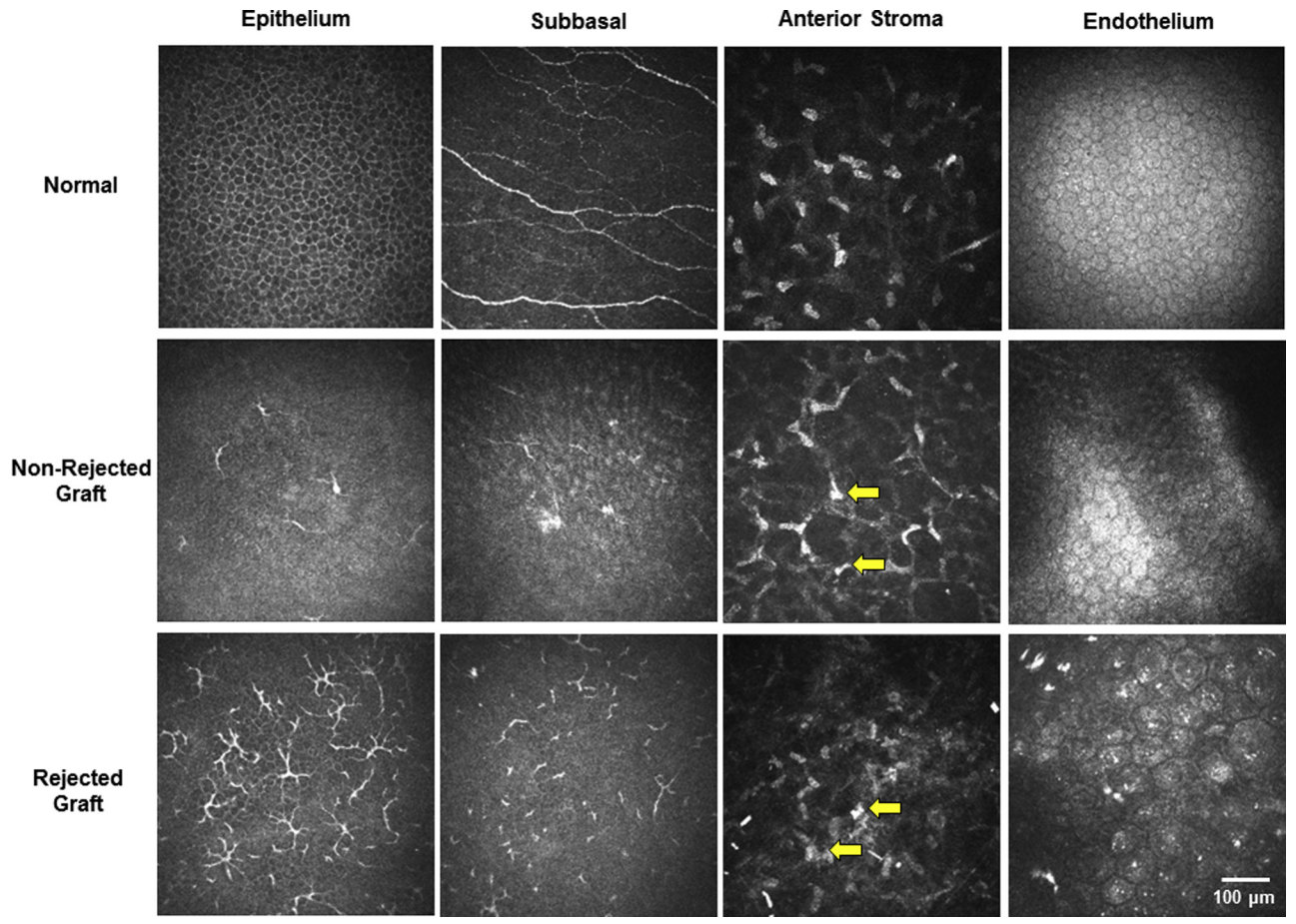

FIGURE 1. In vivo confocal microscopy images of 4 corneal layers: basal epithelium (left column); sub-basal layer (middle-left column); anterior stroma (middle-right column); endothelium (right column), compared among normal controls (row 1), patient with non-rejected graft (row 2), and patient with rejected graft (row 3). Stromal inflammatory cells were identified by manually adjusting image contrast (arrows) and identified by their bean-shaped and irregular nuclei.[31]

the indications for PKP between the two PKP groups ($P > 0.05$). The 2 most common indications for PKP in the non-rejected group were pseudophakic bullous keratoplasty (30.4%; n = 7) and previous graft failure (30.4%; n = 7), whereas the most common indication in the rejected PKP group was keratoconus (40.0%; n = 6). Diabetes mellitus was the only comorbid condition reported in each group, and the number of cases was similar between the groups (n = 4; 10.5% for non-rejected grafts; and 26.7% for rejected grafts). The central corneal thickness in rejected grafts was significantly increased in the rejected PKP group compared to that in the non-rejected PKP group ($P = 0.009$).

Clinical signs and symptoms supported the diagnosis of graft rejection (Table 2). A total of 100.0% of the patients in the rejected group reported decreased vision, and 66.7% of patients reported ocular irritation. Among these patients were 2 who urgently presented before their follow-up appointment due to significantly decreased vision and ocular discomfort. Ciliary injection and corneal edema were the 2 most common signs, occurring in 86.67% of cases with graft rejection. In contrast, none of the patients with non-rejected grafts reported symptoms. Nonetheless, in those patients, ciliary injection occurred in 21.7% of cases, and corneal NV occurred in 4.3% of cases. All signs and symptoms differed significantly ($P < 0.025$) between the rejected and non-rejected PKP groups, except for corneal NV.

• **CORNEAL IMMUNE CELLS DETECTED BY IN VIVO CONFOCAL MICROSCOPY:** The IC density was quantified separately for each corneal layer (Table 3). Significant differences in IC densities were found among normal control patients, non-rejected grafts and rejected grafts in all corneal sublayers ($P < 0.05$). IC densities were significantly higher in both the non-rejected and rejected PKP groups (63.35 ± 12.20 and 95.44 ± 19.75 cells/mm^2, respectively) than in the normal control group (0.24 ± 0.24 cells/mm^2) in the epithelial layer ($P = 0.005$ and $P < 0.001$, respectively) as well

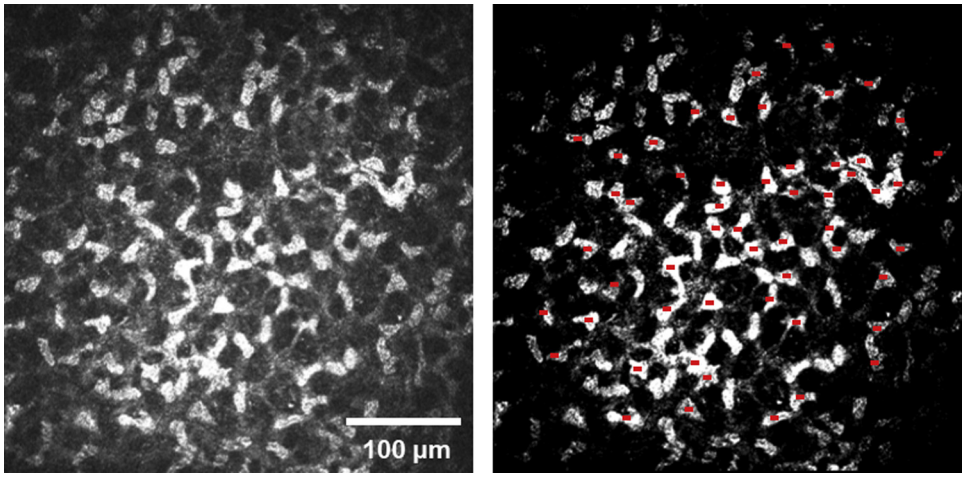

FIGURE 2. Atypical hyper-reflective inflammatory cells in the stromal layer and activated keratocytes were differentiated by manually adjusting image contrast. Inflammatory cells were identified by their bean-shaped nuclei, whereas keratocytes have cigarshaped nuclei with a more regular shape.[31,32] The ICs were distinctly identified by red dots in a patient with acute graft rejection in the anterior stroma. IC = immune cell.

TABLE 1. Characteristics of All Study Participants

	Normal	Non-Rejected Graft	Rejected Graft	*P*
n Participants	9	23	15	
Age (y)	38.14 ± 2.18	56.04 ± 4.59	47.80 ± 5.10	0.09[a]
BCVA at the time of IVCM scan	0.00	0.99 ± 0.15	1.08 ± 0.20	0.85[b]
Pachymetry (µm)	NA	556.44 ± 12.42	713.91 ± 48.67	0.009[b,d]
Indications for corneal transplantation (n, %)	NA			0.25[c]
PBK		7 (30.4)	1 (6.7)	
FECD		0 (0)	1 (6.7)	
Corneal scar		1 (4.3)	0 (0)	
Keratoconus		4 (17.4)	6 (40.0)	
Graft failure		7 (30.4)	3 (20.0)	
Therapeutic keratoplasty				
Necrotizing herpetic keratitis		1 (4.3)	0 (0)	
Bacterial keratitis		1 (4.3)	1 (6.7)	
Fungal keratitis		1 (4.3)	1 (6.7)	
Acanthamoeba keratitis		1 (4.3)	2 (13.3)	

BCVA = best-corrected visual acuity, represented as LogMAR scale; FECD = Fuchs' endothelial corneal dystrophy; IVCM = in vivo confocal microscopy; NA = not assessed; PBK = pseudophakic bullous keratopathy.

Data are means ± SEM, unless otherwise noted. [a]ANOVA; [b]*t*-test; [c]chi square test; [d]$P < 0.05$.

as in the anterior stroma (non-rejected grafts: 54.74 ± 4.84; rejected grafts: 62.62 ± 10.73; controls: 5.39 ± 0.68 cells/mm^2; $P < 0.001$ for both groups). However, the IC densities did not differ significantly between non-rejected and rejected grafts in these 2 layers.

In the sub-basal and endothelial layers, IC densities in patients with rejected grafts (97.84 ± 23.70 and 29.40 ± 10.46 cells/mm^2, respectively) were significantly higher than in patients with non-rejected grafts (54.49 ± 8.96 and 15.11 ± 2.78 cells/mm^2, respectively; $P = 0.049$ and $P = 0.001$, respectively) and controls (42.96 ± 10.94 cells/mm^2; $P = 0.001$; and 0.00 ± 0.00 cell/mm^2, respectively; $P < 0.001$), whereas there were no significant differences in IC densities between normal control and non-rejected grafts. The total IC density was higher in patients with corneal grafts and even higher in rejected grafts than that in normal control grafts. The total IC density differed significantly ($P < 0.001$) among the 3 study groups in ascending order: controls showed 48.60 ± 10.67 cells/mm^2; non-rejected grafts showed 187.70 ± 22.80 cells/mm^2;

TABLE 2. Clinical Signs and Symptoms of Patients with Corneal Transplantation

	Non-Rejected Graft (n = 23)	Rejected Graft (n = 15)	*P*
Symptoms (n, %)			
Decrease in BCVA	0 (0)	15 (100)	<0.001[a]
Irritation	0 (0)	10 (66.7)	<0.001[a]
Light sensitivity	0 (0)	9 (60.0)	<0.001[a]
Pain/pressure	0 (0)	7 (46.7)	<0.001[a]
Signs (n, %)			
Ciliary injection	5 (21.7)	13 (86.7)	<0.001[a]
Epithelial rejection line	0 (0)	5 (33.3)	0.003[a]
Subepithelial infiltrate	0 (0)	0 (0)	-
Stromal edema	0 (0)	13 (86.7)	<0.001[a]
Keratic precipitates	0 (0)	8 (53.3)	<0.001[a]
Khodadoust line	0 (0)	4 (26.7)	0.009[a]
Anterior chamber cells	0 (0)	3 (20.0)	0.025[a]
Corneal neovascularization	1 (4.3)	1 (6.7)	0.75

BCVA = best-corrected visual acuity, shown on the LogMAR scale.
[a]$P < 0.05$.

and rejected grafts showed 285.32 ± 53.23 cells/mm^2 (Figure 3).

Seven of 23 patients with non-rejected grafts underwent repeated IVCM scans at their 1-year post-transplantation follow-up examination. IC densities decreased from the baseline in all corneal layers, and they were significantly lower than those in patients with rejected grafts (all $P < 0.05$) (Table 4).

• **ASSOCIATIONS BETWEEN CORNEAL IMMUNE CELL DENSITY AND CLINICAL SYMPTOMS OF GRAFT REJECTION:** Ocular irritation and light sensitivity were associated with significant increases in IC densities in the epithelial and sub-basal layers ($P < 0.05$). Ocular pain was associated significantly with increased IC density only in the epithelium ($P = 0.03$). No symptoms correlated to increases in IC density in the anterior stroma or endothelial layer (Supplemental Table 2). However, all clinical symptoms were correlated significantly with increased total IC density ($P < 0.05$) (Table 5).

• **ASSOCIATIONS BETWEEN CORNEAL IMMUNE CELL DENSITY AND CLINICAL SIGNS OF GRAFT REJECTION:** One or more typical signs of corneal graft rejection (ie, ciliary injection, epithelial rejection line, stromal edema, KPs, Khodadoust line, AC cells, and corneal NV) were observed in all patients with rejected grafts. In contrast, the only 2 clinical signs detected in patients with non-rejected grafts were ciliary injection and corneal NV. The IC densities in all corneal layers were analyzed and compared between patients with and without clinical signs of rejection. Increased IC densities in specific corneal layers were associated with specific signs of corneal graft rejection (Table 6). Corneal NV was strongly correlated with significantly increased IC density in the epithelial ($P < 0.001$) and sub-basal layers ($P = 0.04$), whereas other clinical signs did not correlate with increased IC densities in the other corneal layers. Significant increases in the total IC densities were seen specifically in patients with Khodadoust lines ($P < 0.009$) and AC cells ($P < 0.001$). Multiple regression analysis was performed next to determine which clinical factors were independently correlated with IC densities. After multiple adjustments for clinical signs and symptoms, light sensitivity remained independently associated with epithelial IC density ($\beta = 0.45$ and $P = 0.004$), sub-basal IC density ($\beta = 0.54$ and $P < 0.001$), and total IC density ($\beta = 0.55$ and $P = 0.001$).

DISCUSSION

THE CURRENT STUDY ANALYZED THE IMMUNE RESPONSE IN patients with corneal allograft rejection by detecting IC densities in each corneal layer of rejected grafts, using laser IVCM and comparing the results with those of non-rejected grafts and age- and sex-matched normal controls. Increased IC densities were found in all layers of clinically diagnosed rejected grafts compared to normal corneas. Among patients who underwent PKP, the IC densities were significantly higher in the sub-basal and endothelial layers of rejected grafts than those in non-rejected grafts. Our findings suggest that increased IC densities, particularly in the sub-basal and endothelial layers of the corneal grafts, may suggest allograft rejection.

Currently, the gold standard method for diagnosing corneal graft rejection is based solely on clinical signs and symptoms.[34] Most patients present with at least 1 of the clinical signs of an inflammatory reaction in their grafts, as seen on slit-lamp examination. Common symptoms of graft rejection are decreased vision, ocular irritation, light sensitivity, and ocular pain. However, other ocular conditions in patients with corneal transplants, such as herpetic keratouveitis, epithelial downgrowth, or early corneal infections can mimic rejection, which can lead to a missed or delayed diagnosis or a dilemma for clinicians.[35] Routine eye examinations using slit-lamp biomicroscopy can allow for assessment of graft rejection only in cases with obvious manifestations, and subtle changes in very early stages of rejection are difficult to detect with a slit-lamp examination. Advanced technologies have been introduced to aid diagnosis and early detection, such as specular microscopy, which was shown to demonstrate morphological changes in the endothelial cells in cases of impending allograft rejection.[19,20] Furthermore, Abou Shousha and associates[22] reported the

TABLE 3. Comparison between Immune Cells Detected by IVCM in Normal Subjects and Those in Patients Who Have Undergone Corneal Transplantation

	Normal	Non-Rejected Graft	Rejected Graft	*P* (ANOVA)*
Epithelium	0.24 ± 0.24[a,b]	63.35 ± 12.21[a]	95.45 ± 19.75[b]	0.001
Sub-basal	42.97 ± 10.94[a]	54.50 ± 8.96[b]	97.85 ± 23.70[a,b]	0.049
Anterior stroma	5.39 ± 0.68[a,b]	54.74 ± 4.84[a]	62.62 ± 10.74[b]	<0.001
Endothelium	0.00 ± 0.00[a]	16.56 ± 2.86[b]	40.10 ± 12.89[a,b]	0.001
Total	48.60 ± 10.68[a,b]	188.14 ± 22.40[a,c]	340.88 ± 64.07[b,c]	<0.001

IVCM = in vivo confocal microscopy.

Data are means ± SEM (cells/mm^2).

Similar superscript letters indicate significant differences among the groups, assessed by least-significant difference post hoc test. Superscript letters a, b, and c represent each pair of significantly different comparisons.

*$P < 0.05$.

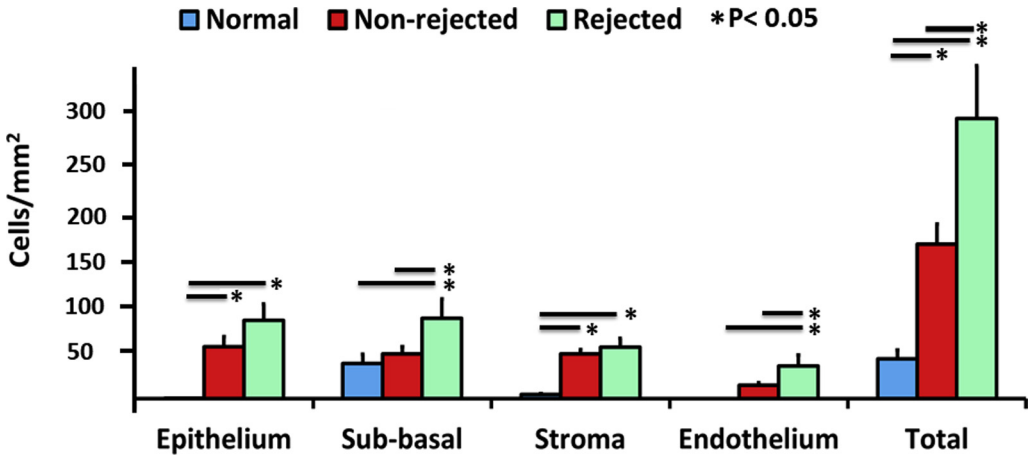

FIGURE 3. Comparison of immune cells detected by in vivo confocal microscopy in each corneal layer between patients who underwent corneal transplantation and normal subjects.

advantage they found in using AS-OCT to assess the thickness of endothelial/Descemet's membrane complex to predict corneal graft rejection. Recently, IVCM has been used widely to assess corneal and ocular surface diseases, and the technology enables visualization of microstructural changes including pathogenic organisms and immune and inflammatory cells present in the cornea.[23–26,36–38] In the current study, we used high-resolution laser IVCM to investigate microscopic changes that enabled differentiation between normal grafts and immunological graft rejection and found that the IC density in rejected grafts increased in all corneal sublayers, particularly in the subbasal and endothelial layers, compared to non-rejected grafts.

Niederer and associates[28] reported 2 cases of patients with clinical graft rejection that demonstrated aggregation of dendrite-like inflammatory cells in the basal epithelium and small deposits in the endothelial layer. However, the data in those 2 patients were limited, and they did not find any alteration of IC densities in other corneal layers as demonstrated in our study. Thus, to the best of our knowledge, this is the first IVCM study that systematically analyzed IC densities in non-rejected and rejected corneal

TABLE 4. Comparison between Immune Cells Detected by IVCM in Patients with Corneal Transplantation Non-Rejected Grafts at 1-Year Post-Transplantation vs Those Who Had Rejected Grafts

	Non-Rejected Graft (1 y) (n = 7)	Rejected Graft (n = 15)	*P*
Epithelium	12.13 ± 4.05	95.45 ± 19.75	0.001[a]
Sub-basal	22.53 ± 5.53	97.85 ± 23.70	0.007[a]
Anterior stroma	28.78 ± 3.04	62.62 ± 10.74	0.008[a]
Endothelium	8.99 ± 3.79	40.10 ± 12.89	0.040[a]
Total	74.84 ± 8.16	340.88 ± 64.07	0.002[a]

IVCM = in vivo confocal microscopy.

Data are means ± SEM (cells/mm^2).

[a]$P < 0.05$.

TABLE 5. Comparison of Immune Cells Detected by IVCM in Patients Who Underwent Corneal Transplantation to Presence and Absence of Clinical Symptoms

Sign	Presence	Epithelium	Sub-basal	Stroma	Endothelium	Total
Decreased vision	No	63.35 ± 12.21	54.50 ± 8.96	54.74 ± 4.84	16.55 ± 2.86	188.14 ± 24.40
	Yes	95.45 ± 19.75	97.85 ± 23.70	62.62 ± 10.74	40.10 ± 12.89	340.88 ± 64.07
	P	0.152	0.104	0.511	0.102	0.044[a]
Irritation	No	59.13 ± 10.36	50.64 ± 7.72	53.18 ± 4.07	20.97 ± 4.93	189.93 ± 22.03
	Yes	123.31 ± 24.75	130.32 ± 30.53	70.94 ± 15.57	35.68 ± 14.00	392.79 ± 79.64
	P	0.008[a]	0.029[a]	0.295	0.348	0.039[a]
Light sensitivity	No	59.35 ± 10.25	50.95 ± 7.63	52.49 ± 3.98	21.39 ± 4.95	187.99 ± 22.34
	Yes	129.72 ± 25.65	138.19 ± 32.53	75.14 ± 16.76	34.43 ± 14.12	398.60 ± 76.58
	P	0.004[a]	0.028[a]	0.222	0.406	0.029[a]
Pain/pressure	No	65.12 ± 11.39	56.80 ± 9.66	54.35 ± 4.57	23.11 ± 4.88	205.50 ± 28.63
	Yes	124.29 ± 24.85	137.19 ± 35.16	73.35 ± 19.00	31.33 ± 18.13	392.94 ± 74.40
	P	0.033[a]	0.064	0.365	0.537	0.011[a]

IVCM = in vivo confocal microscopy.
Data are means ± SEM.
[a]$P < 0.05$.

TABLE 6. Comparison of Immune Cells Detected by IVCM in Patients with Corneal Transplantation to Presence and Absence of Clinical Signs

Sign	Presence	Epithelium	Sub-basal	Stroma	Endothelium	Total
Keratic precipitates	No	68.55 ± 11.06	61.98 ± 10.26	56.23 ± 5.23	17.03 ± 2.37	203.48 ± 27.05
	Yes	104.04 ± 30.58	107.71 ± 35.57	63.94 ± 14.94	57.66 ± 21.60	401.69 ± 82.27
	P	0.188	0.251	0.545	0.119	0.007[a]
Khodadoust line	No	72.82 ± 10.69	66.21 ± 10.20	55.84 ± 5.36	25.35 ± 5.41	221.03 ± 27.92
	Yes	103.24 ± 54.12	117.51 ± 64.19	75.00 ± 15.93	14.06 ± 2.81	534.87 ± 59.87
	P	0.617	0.485	0.255	0.600	0.008[a]
Corneal NV	No	71.49 ± 11.01	66.32 ± 10.88	55.19 ± 4.66	24.94 ± 5.42	226.66 ± 29.52
	Yes	157.50 ± 4.37	166.87 ± 60.75	105.78 ± 47.34	20.23 ± 8.20	450.39 ± 95.52
	P	<0.001[a]	0.043[a]	0.478	0.827	0.066

IVCM = in vivo confocal microscopy; NV = neovascularization.
Data for significant clinical signs are means ± SEM.
[a]$P < 0.05$.

grafts. Our results suggest that IVCM may aid as an adjunct tool in the diagnosis of corneal graft rejection, particularly in patients with subtle manifestations.

The increase in IC density we found in rejected grafts may be due to allogeneic graft rejection, in which various inflammatory cells (neutrophils, macrophages, and lymphocytes) are recruited to the graft tissue.[39] Diffuse inflammation throughout the entire graft results in endothelial damage and subsequent graft failure. Based on evidence shown in published reports, the most commonly reported cause of corneal graft failure is endothelial rejection. Thus, we had hypothesized accumulation of immune and inflammatory cells in the endothelial layer in this study.[40] However, more than 1 corneal layer may simultaneously be involved during the rejection process.[17, 41] Accordingly, we found a significant increase in total IC density in rejected grafts compared to that in non-rejected grafts and controls.

When we compared the IC densities between rejected and non-rejected grafts layer by layer, significantly higher IC densities were seen only in the sub-basal and endothelial layers of rejected grafts than in non-rejected grafts. However, when 7 patients in the non-rejected group underwent repeated IVCM scans 1 year after transplantation, the IC densities in all 4 corneal layers decreased from the those measured at the 1-month follow-up and were significantly ($P < 0.05$) lower than in patients with rejected grafts.

These results and overall increase in IC density in the grafts helped to differentiate between rejected and non-rejected corneal grafts.

We found correlation between clinical symptoms and total IC density in patients with corneal graft rejection. IC density was significantly higher in the epithelial and sub-basal layers in patients with symptomatic ocular irritation and light sensitivity. We further analyzed our data to determine whether our clinical measurements were independently correlated with IC densities. After performing multiple regression analysis, light sensitivity remained independently associated with epithelial, sub-basal, and total IC densities. Corneal nerves are abundant throughout the epithelial and sub-basal layers and contain multiple sensory fibers that respond to pain and thermal and mechanical pressure.[42] Infiltration of ICs in the epithelium and sub-basal layers may result in an increase of inflammatory mediators, such as cytokines, and may result in various manifestations of ocular discomfort.[43] Additionally, accumulation of ICs in any corneal layer can disrupt the organized collagen fibrils and cause loss of corneal transparency and decreased vision. Accordingly, we found a significant increase in the total IC density in patients who reported decreased vision.

Our data indicate a trend toward elevated IC density in every corneal layer in patients with clinical signs of corneal graft rejection, especially those with AC cells and a Khodadoust line. These findings indicate that the clinical signs used to diagnose graft rejection are likely related to an inflammatory reaction in rejected grafts. We found that pathognomonic signs such as the Khodadoust line and the presence of AC cells, both a result of drastic immune reactions, are associated with a significant increase in the total numbers of ICs in such grafts.

Although advanced surgical techniques and postoperative medications for corneal transplantation have been developed and appear to improve visual outcomes, a major concern with all keratoplasties is allograft rejection, which could eventually cause graft failure.[44–46] Early diagnosis and prompt therapy may help improve the success rate of corneal graft rejection treatment and increase the likelihood of long-term graft survival. Unfortunately, some diagnostic signs may manifest only when the immunologic reaction intensifies and becomes more advanced, leading to delayed diagnosis and treatment. In the current study, we demonstrated the use of IVCM to detect ICs inside corneal grafts during the rejection process. Increased numbers of ICs agreed with the clinical signs and symptoms, indicating that IVCM may be useful in supporting the diagnosis of graft rejection, especially in questionable cases. Moreover, the identification of potential predictors for keratoplasty outcomes or antirejection therapy has been a long-desired goal. Current studies are underway by our group to assess whether imaging biomarkers by IVCM could provide such measures for patients with corneal transplantations and other corneal diseases, which also include deep machine learning algorithms for these purposes.

In routine clinical practice, the diagnosis of corneal graft rejection may be challenging. In particular, herpetic keratouveitis or early corneal infections may mimic graft rejection. In fact, these conditions may be accompanied by clinical inflammation and, thus, may result in subsequently increased IC density by IVCM. However, there are some specific characteristic findings by IVCM that may assist in differentiating these conditions from graft rejection. Previous studies have reported specific IVCM findings in patients with HSV keratouveitis, including the presence of pseudoguttata, enlarged intercellular gaps, loss of distinct endothelial cell boundaries, spot-like holes, deceased corneal nerve density, and decreased endothelial cell density.[47–50] Detection of epithelial cells posterior to the corneal endothelial layer has been presumed to be a specific finding in cases of epithelial downgrowth,[51] whereas specific shape and focal changes of dendritiform IC cells in the subepithelial layer, which depend on pathogenic organisms, have been shown in infectious keratitis.[52] Given that none of those IVCM findings were detected in our patients, one could speculate that diffuse increase in IC density as shown in the current study could be associated with early graft rejection.

The limitations of the current study included the area of grafts imaged and the timing of IVCM scanning. We selected the central area for analysis to ensure practical and consistent measurements across patient groups. Furthermore, we also expected that there should have been some degree of central corneal inflammation in rejected grafts. This was found to occur, as we observed corneal edema (relatively high central corneal thickness) in every patient with graft rejection. Other studies of corneal allograft rejection generally have considered changes in the central graft area, including the central corneal thickness.[21,53,54] However, rejection can occur anywhere on the graft before emanating throughout the entire cornea. Multiple scans in different areas of the corneal grafts would provide more substantial results. Regarding the time point of IVCM imaging, patients with non-rejected grafts underwent IVCM scanning in their early postoperative period (4–8 weeks after keratoplasty). The inflammation induced in the early postoperative period was expected to be higher in response to the wound healing process,[55] which predominated in the epithelial and stromal layers.[56,57] Therefore, the number of ICs in the epithelium and stroma of non-rejected grafts in this study may be enhanced by this mechanism results in non-significant difference of ICs number in these 2 layers between rejected and non-rejected grafts, albeit when compared to controls, there were significant differences in all layers for ICs. When we compared the IC densities rejected grafts with those

in non-rejected grafts, significantly increased ICs were seen only in the sub-basal and endothelial layers. Interestingly, IC densities of non-rejected grafts at the 1-year follow-up examination were decreased from the early post-transplantation time point and were significantly lower in all corneal layers than in rejected grafts. We believe that a prolonged use of topical steroid and resolution of postoperative inflammatory response may play roles in this reduction of IC numbers in these grafts.[58] Future longitudinal studies are warranted to potentially identify a cutoff point of IC density in each corneal layer that could be applicable as a diagnostic criterion for graft rejection.

In conclusion, increased IC density in corneal grafts detected by IVCM, particularly in the sub-basal and endothelial layers, represented an immune reaction of corneal graft rejection. IVCM may be used to support the diagnosis of corneal graft rejection.

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