

# Ocular Inflammation in HLA-B27 Transgenic Mice Reveals a Potential Role for MHC Class I in Corneal Immune Privilege

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**Purpose:** HLA-B27 is a major histocompatibility complex class I (MHCI) allele that has been closely associated with the development of ankylosing spondylitis and acute anterior uveitis (AAU), the most common form of uveitis worldwide. We have been characterizing the phenotypes of transgenic mice carrying a human HLA-B27 allele, but that are deficient in endogenous mouse MHCI genes (H-2K<sup>-/-</sup> and H-2D<sup>-/-</sup> double knockout, or DKO) to create the HLA-B27/DKO line. In maintaining and expanding this colony, we observed a rare sporadic severe central keratitis that developed in transgenic animals, but that was not present in wild-type (WT) animals.

**Methods:** The corneas of affected HLA-B27/DKO and DKO mice were compared to their WT counterparts by staining with standard histological methods for markers of inflammation and neovascularization. A model of experimental corneal inflammation was subsequently used to test the responses of each genotype to insult.

**Results:** We identified a previously unreported corneal pathology in naive HLA-B27/DKO mice, and we describe significantly prolonged CD4<sup>+</sup>- and CD8<sup>+</sup>-associated inflammation in these animals following an experimentally induced corneal injury.

**Conclusions:** These results demonstrate an increased T-cell response in B27/DKO corneas due to the expression of the HLA-B27 allele, suggesting that low MHCI expression in WT corneas is an important contributor to immune privilege.

Human leukocyte antigens (HLAs) are encoded by major histocompatibility complex class I (MHCI) genes, which play a major antigen presentation role in the adaptive immune system. As such, there are a large number of HLA subtypes and alleles that provide extensive genetic diversity to host immunity [1]. HLA-B27, an MHCI molecule responsible for antigen presentation to CD8<sup>+</sup> T lymphocytes, has been closely associated with the development of ankylosing spondylitis and associated spondyloarthropathies [2,3]. Notably, HLA-B27 is also associated with the development of acute anterior uveitis (AAU), the most common form of uveitis worldwide [4,5]. In North America, the prevalence of the HLA-B27 allele in AAU patients is around 50% [6-8], and it is the most common genetic marker associated with the development of AAU [5,7,9]. This AAU is typically unilateral with substantial cellular and protein extravasation into the anterior chamber. Previous studies with HLA-B27 transgenic mice and rats have reproduced aspects of systemic spondyloarthritis [10-12]. However, evidence of AAU in these animals has generally been mild or negligible.

To study the role of HLA-B27 in disease, we have been characterizing the phenotypes of HLA transgenic mice. These animals were generated by crossing a transgenic strain carrying a human HLA-B27 allele with mice deficient in the endogenous mouse MHC class I genes, H-2K<sup>-/-</sup> and H-2D<sup>-/-</sup> (double knockout or DKO), to create the HLA-B27/DKO line [13,14]. In maintaining and expanding this mouse colony, a large number of transgenic and wild-type (WT) animals were generated. During this work, we observed a rare sporadic severe central keratitis that developed in transgenic animals, but that was not present in WT animals. This previously unreported phenotype was observed most often in HLA-B27/DKO animals and occasionally in DKO animals, but never in non-transgenic WT mice. Here we present our characterization of this pathology in naive animals, and following experimentally induced corneal inflammation.

## METHODS

**Transgenic mice:** The HLA Tg B27 mouse strains were generated and described in detail previously [13]. The HLA Tg B27 mice on the C57BL/6 background were subsequently backcrossed with mice deficient in murine endogenous H2 class I (H2-K<sup>-/-</sup>D<sup>-/-</sup> [DKO] mice) at least six times to generate HLA Tg B27/DKO strains [14]. HLA Tg B27/DKO and DKO offspring were categorized by flow cytometry of PBLs. HLA Tg B27/DKO was detected by monoclonal antibody (mAb)

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ME1 and mAb BB7.1. DKO was demonstrated using mAb 28–6-s. The mAbs used for flow cytometry were from the American Type Culture Collection (Manassas, VA), and the FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse IgG (Fc-specific) was from Jackson ImmunoResearch Laboratories (West Grove, PA). The C57BL6 (WT) mice were used as a control in this study. All mice were housed in the specific pathogen-free animal facility at Toronto Western Hospital in Toronto according to the guidelines of the Canadian Council of Animal Care. All animal studies were reviewed and approved by the University Health Network Research Committee.

**Corneal debridement model:** Corneal neovascularization was induced in mice between 6–8 weeks old through the transient removal of the corneal epithelium by gentle mechanical scraping, as previously described [15]. Briefly, mice were anesthetized by an i.p. administration of 250 mg/kg avertin. All eyes were locally anesthetized by a topical application of a 0.5% proparacaine solution (Bausch and Lomb, Rochester, NY) for 1 min. A topical anesthetic was blotted away with sterile gauze. Sterile PBS was applied to keep the eyes moist during surgery, which was performed under a standard laboratory dissecting microscope. The eyes were proptosed with serrated forceps, and the corneal epithelium was removed with a sterile disposable scalpel using central brushing motions following the corneal surface. An antibiotic ointment was applied to the debrided eyes and the animals were allowed to recover on a warming pad. Animals were then returned to the colony for 7 or 14 days, as indicated, before euthanasia and histological analyses.

**Embedding and sectioning:** To analyze the immune response of the cornea, animals were sacrificed on days 7 and 14 after scraping. Animals were euthanized with Ketamine/Xylazine followed by cervical dislocation. Eyes were enucleated with curved tweezers, slightly rinsed in 1 X PBS, and fixed with 4% paraformaldehyde at 4 °C overnight under constant rocking. After fixation, eyes were washed with 1 X PBS and equilibrated in 30% sucrose solution at 4 °C until the samples sank to the bottom of the container. Eyes were then frozen in optical cutting temperature (OCT) (Sakura, Torrance, CA) on dry ice, and serial sections were cut at a thickness of 10 µm, mounted on SuperfrostPlus slides (VWR, Radnor, PA), and stored at –80 °C.

**Histology and Immunofluorescence staining:** For histology, sections were stained with hematoxylin and eosin (H&E), according to established protocols. For immunofluorescence, the sections were air-dried at room temperature for 15 min and with 4% paraformaldehyde for 10 min. Sections were then rinsed with 1 X PBST (0.25% Triton-X100). Blocking was performed for 30 min at room temperature in 5% normal

serum in PBS. Sections were incubated with primary antibodies diluted in PBS for 2 h at room temperature. Sections were washed with 1 X PBS and incubated with secondary antibodies diluted in PBS for 1 h at room temperature. After washing again, sections were mounted with the VECTA-SHIELD HardSet mounting medium with DAPI (Vector Laboratories, Burlingame, CA) and imaged. Primary antibodies used were: rat anti-mouse Ly6G and rat anti-mouse F4/80 (BioLegend, San Diego, CA), rat anti-mouse CD31, rat anti-mouse CD4, and rat anti-mouse CD8a (BD PharMingen, Mississauga, ON). Alexa Fluor 546, goat anti-rat (Life Technology, Burlington, ON) was used as the secondary antibody. All histology and immunofluorescence images are representative of *at least* three independent animal experiments.

**Scoring and statistics:** Scoring of keratitis was determined from H&E slides in a blinded fashion. A score of '0' was assigned when there was no evidence of infiltrates or morphological disruption, '1' if a few infiltrates were present, '2' if a moderate number of infiltrates were present along with mild tissue damage, and '3' if there was extensive damage, swelling, and a great number of infiltrates. To compare the resolution of corneal inflammation between genotypes, the difference between days 7 and 14 post-debridement was analyzed by a non-parametric Mann–Whitney U test. Differences were considered significant at p value ≤0.05. A statistical analysis was performed using the GraphPad Prism software (version 5.00).

## RESULTS AND DISCUSSION

**DKO and B27/DKO mice develop sporadic severe central corneal keratitis:** During colony breeding, we observed that a severe cloudy opacity occasionally developed in the central cornea of B27/DKO animals (6%; 6 of 106 animals), rarely in DKO animals (2%; 2 of 75 animals), and never in WT animals (0%; 0 of 70 animals). The opacity appeared to develop sporadically by three weeks of age, often presenting bilaterally with a variable pathology in each eye. There was no clear evidence of an aqueous flare or purulent discharge observed (Figures 1A, B). However, because of the established links between HLA-B27 and anterior uveitis, we followed up with this observation to characterize the phenotype further. Eyes from the affected animals were enucleated, fixed, and then sectioned for histopathological assessment.

Sections stained with H&E revealed a deep stromal keratitis in the central cornea, characterized by robust swelling, inflammatory infiltrates, and extensive vascularization (Figure 1D). Though irregular, the epithelium generally remained intact over this region, but the endothelium was often disrupted. Toward the limbus, the general tissue

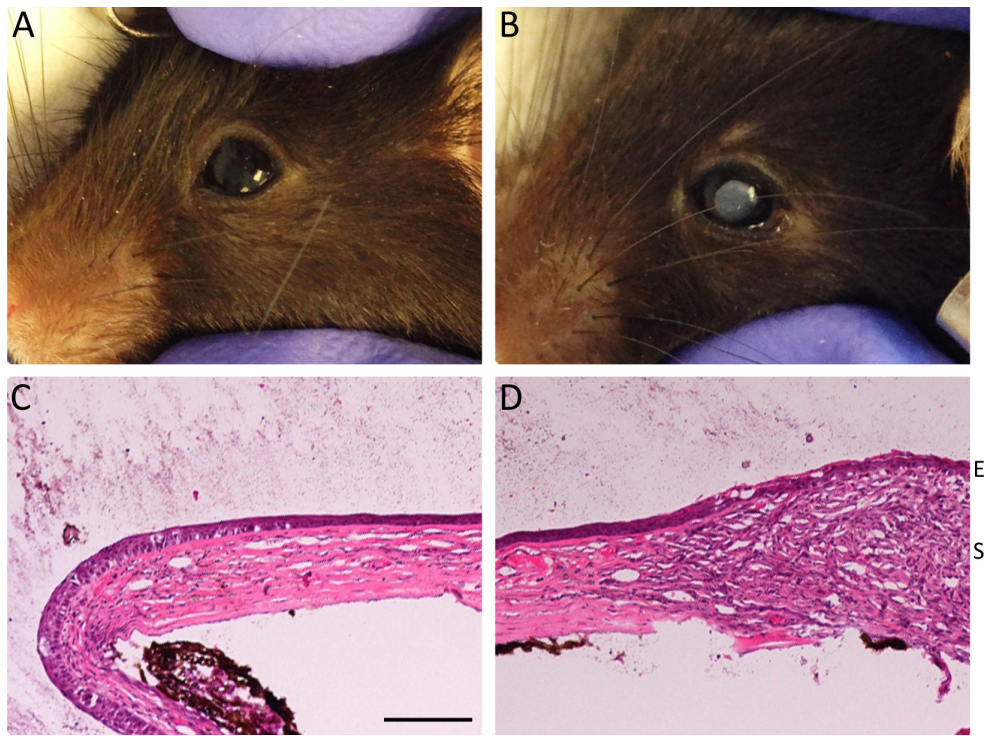


Figure 1. Rare corneal opacities observed in HLA-B27/DKO mice. **A:** A clear eye from an HLA-B27/DKO mouse. **B:** The contralateral eye developed a severe central corneal opacity. **C:** H&E staining of the periphery of an affected cornea shows some inflammatory infiltrates but limited tissue disruption. **D:** H&E staining of the central cornea shows a damaged but intact epithelium and a severely disrupted stroma, accompanied by a massive number of tissue infiltrates and neovascularization (n = 6). (**E:** epithelium, **S:** stroma, bar indicates 50  $\mu$ M).

morphology was preserved, but milder evidence of infiltrates and neovascular growth was observed (Figure 1C). As it is unusual for inflammation to dominate the central cornea rather than the periphery, an additional characterization was warranted.

For a more precise characterization, neighboring sections were also stained with antibodies raised to markers of inflammation and neovascularization and assessed by immunofluorescence microscopy. The central regions of the affected corneas were prominently stained by antibodies raised to the vascular endothelial marker CD31, CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, macrophages (F4/80), and neutrophils (Ly-6G; Figure 2). These results suggest a broad inflammatory response is engendered in the central corneal stroma, which is accompanied by robust neovascularization.

*Induced corneal inflammation is prolonged in HLA-B27/DKO animals:* The central keratitis phenotype was sporadic in HLA-B27/DKO animals, and the trigger remained unknown. However, the healthy cornea typically exhibits a low expression of MHC class I genes, a characteristic that contributes to its immune privilege [16,17]. Due to the ubiquitous expression of HLA-B27 in this transgenic strain, we hypothesized that an excessive corneal T-cell response may result from injury or infection. Therefore, we designed a follow-up study to induce a broad corneal inflammatory response as an attempt to deliberately trigger the keratitis phenotype.

For this purpose, we used a debridement model of corneal inflammation and angiogenesis that we have previously described [15]. Following established methods, the corneal epithelium was removed from the left eye of animals of each genotype by gentle debridement with a scalpel blade. Typically, this procedure results in rapid inflammation characterized by extensive neutrophil and macrophage infiltration and angiogenesis, as well as a generally mild T-cell response [15,18,19]. The epithelium resurfaces within 2–3 days, and the inflammation begins to resolve after one week, although the new blood vessels remain [15]. We therefore assessed the debrided corneas at two time points in each strain: after 7 days, when the inflammatory response should still be high, and after 14 days, when WT corneas would normally resolve.

H&E staining of the sections from WT animals revealed the expected infiltrates in the central corneal stroma, accompanied by mild swelling at day 7 (Figure 3A). By day 14, both the swelling and infiltrates had generally resolved (Figure 3B). In comparison, DKO and B27/DKO animals exhibited increasingly exacerbated inflammation and swelling at day 7 (Figures 3C, E). Furthermore, B27/DKO animals often clearly showed sustained infiltrates that did not resolve at day 14 (Figure 3F), as compared to WT and DKO (Figures 3B, D) animals. These results were quantified by blinded scoring of H&E-stained cornea sections from multiple animals of each genotype. Statistical analyses indicated there was a consistent

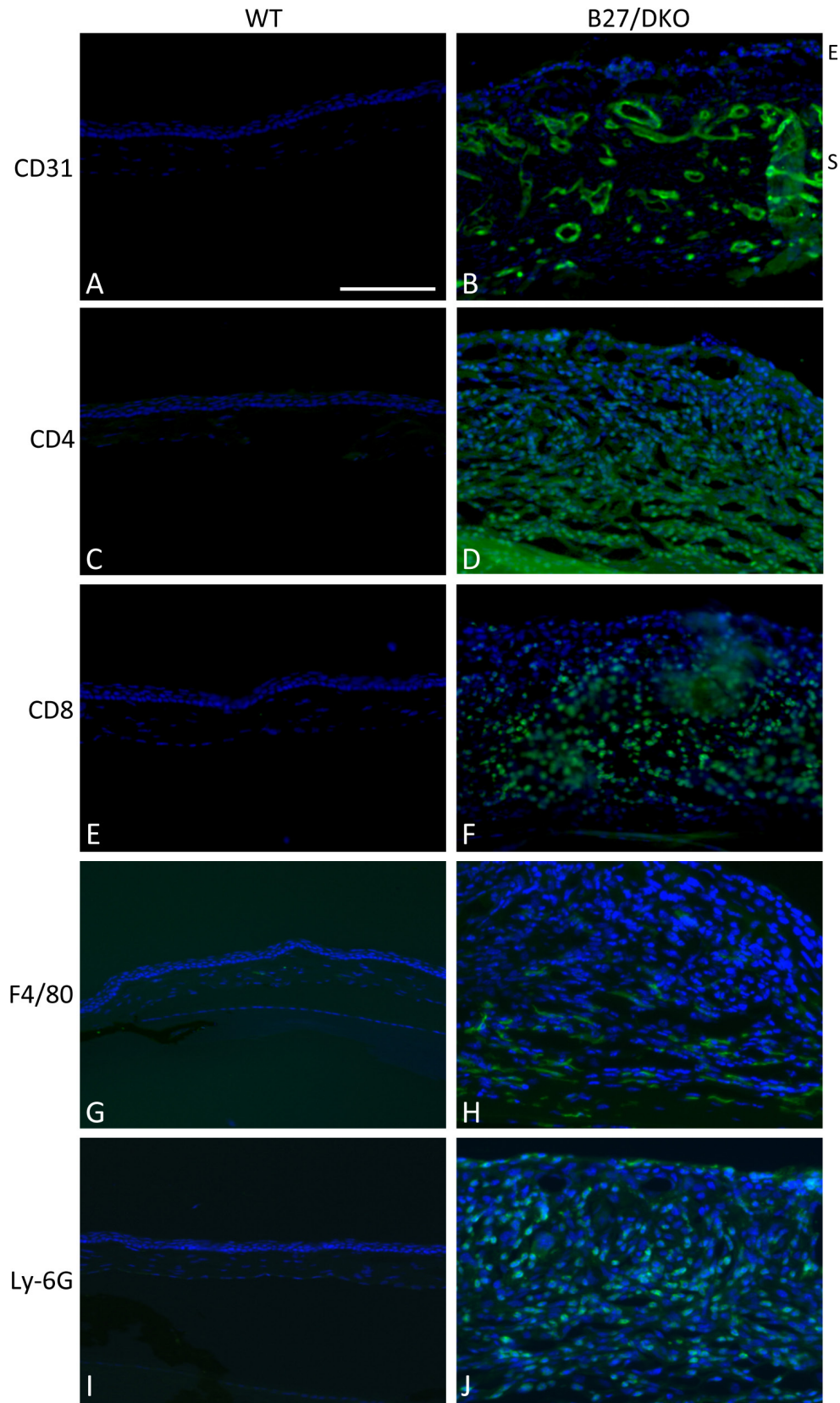


Figure 2. Broad inflammation and neovascularization characterize affected corneas. Central corneas from affected HLA-B27/DKO animals stain positive for a panel of markers, including CD31 (A, B), CD4 (C, D), CD8 (E, F), F4/80 (G, H), and Ly-6G (I, J), as compared to negative WT controls (n = 6). (Bar indicates 50  $\mu$ M).

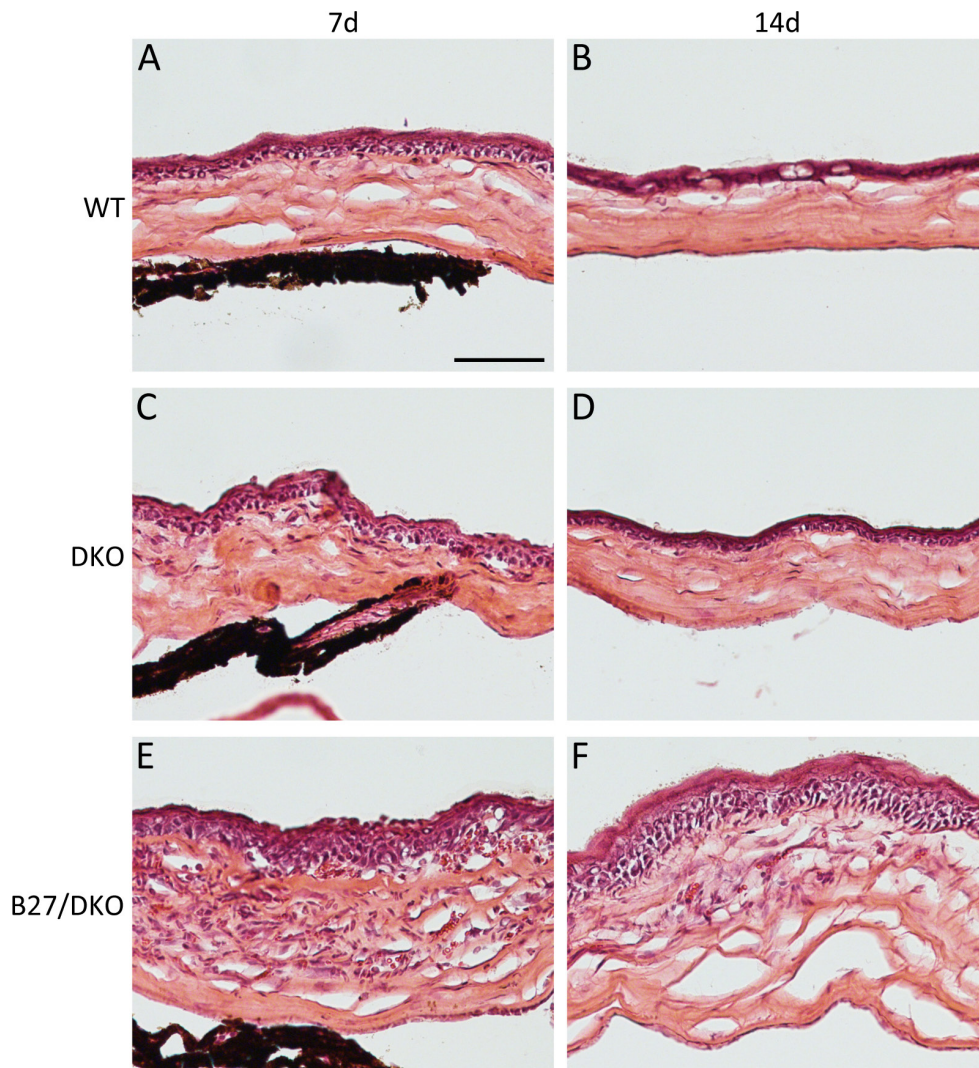


Figure 3. HLA-B27/DKO corneas are deficient in resolving induced inflammation. H&E-stained central corneas from debrided WT, DKO, and HLA-B27/DKO animals all show tissue swelling and infiltrates after 7 days (7d, panels A, C, E). After 14 days (14d), WT and DKO corneas had resolved, but HLA-B27/DKO corneas had not (panels B, D, F).

trend toward increased inflammation in DKO and B27/DKO animals at days 7 and 14 compared to WT animals, but this change did not reach significance ( $p > 0.05$ ). More strikingly, there was a significant resolution of inflammation in WT and DKO animals at day 14, but the inflammation persisted in B27/DKO animals (Figure 4).

As MHC class I HLAs are involved in T-cell antigen presentation, we hypothesized that the constitutive expression of HLA-B27 in transgenic corneas would stimulate a robust and persistent T-cell response during debridement-induced inflammation. Sections from animals at 14 days post-debridement were therefore stained with antibodies raised to CD4 and CD8. Central corneas from WT and DKO animals did not exhibit strong staining for either marker (Figures 5A–B, D–E). In particular, DKO animals have been reported to exhibit markedly deficient CD8<sup>+</sup> responses, which may contribute to the exacerbated inflammation observed in

these animals, possibly due to the increased activity of other T-cell populations [13,20]. However, corneas from B27/DKO animals exhibited robust and persistent staining for CD4<sup>+</sup> and CD8<sup>+</sup> cells (Figures 5C, F).

Therefore, these results are consistent with an increased T-cell response in B27/DKO corneas due to the expression of the HLA-B27 MHC class I allele, particularly the induction of a persistent CD8<sup>+</sup> response that is deficient in DKO and resolves in WT animals. Previous evidence has suggested instead that CD4<sup>+</sup> and CD8<sup>+</sup> allograft recognition is primarily mediated through MHC class II molecules [16]. Therefore, an improved understanding of the factors regulating the MHC class I expression in the cornea may provide additional insight into certain types of graft failures [21-23].

Additionally, several new questions are generated by these results that will require further study. The explanation

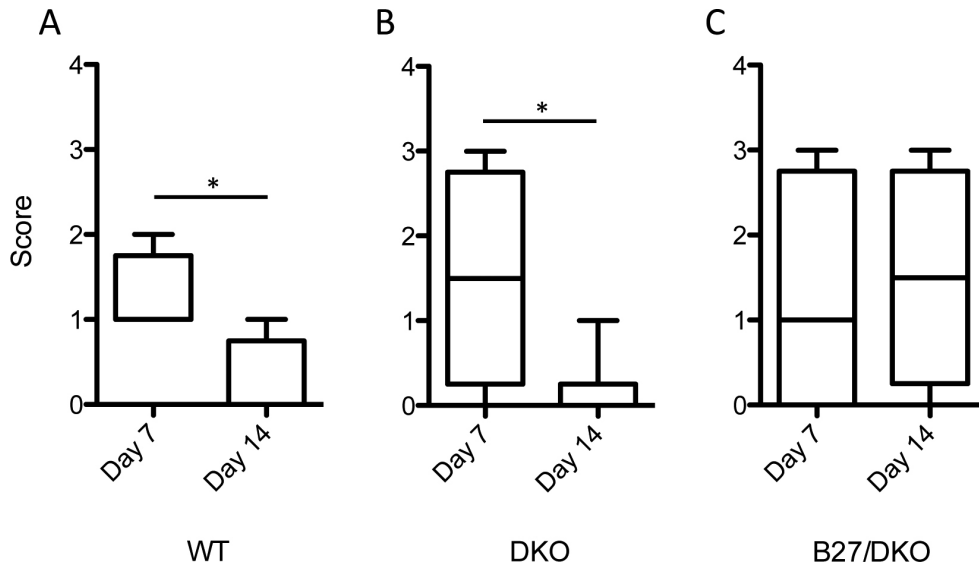


Figure 4. Persistent inflammation in HLA-B27/DKO corneas. Box and whisker plots of corneal inflammation scores from H&E-stained sections of WT, DKO, and HLA-B27/DKO animals following corneal debridement. **A:** WT and **B:** DKO corneas were significantly resolved by day 14 but **C:** HLA-B27/DKO corneas did not. (n = 4 animals, \*p<0.05).

for the unusual development of a central keratitis remains unclear; is it mediated by particular immune cell populations that are suppressed near the limbal vasculature? In addition, as the trigger(s) for the sporadic keratitis in our B27/DKO

animals remains unknown, an additional characterization of the B27/DKO genotype may reveal sensitivities for particular pathogens that do not induce a strong immune response in the general population.

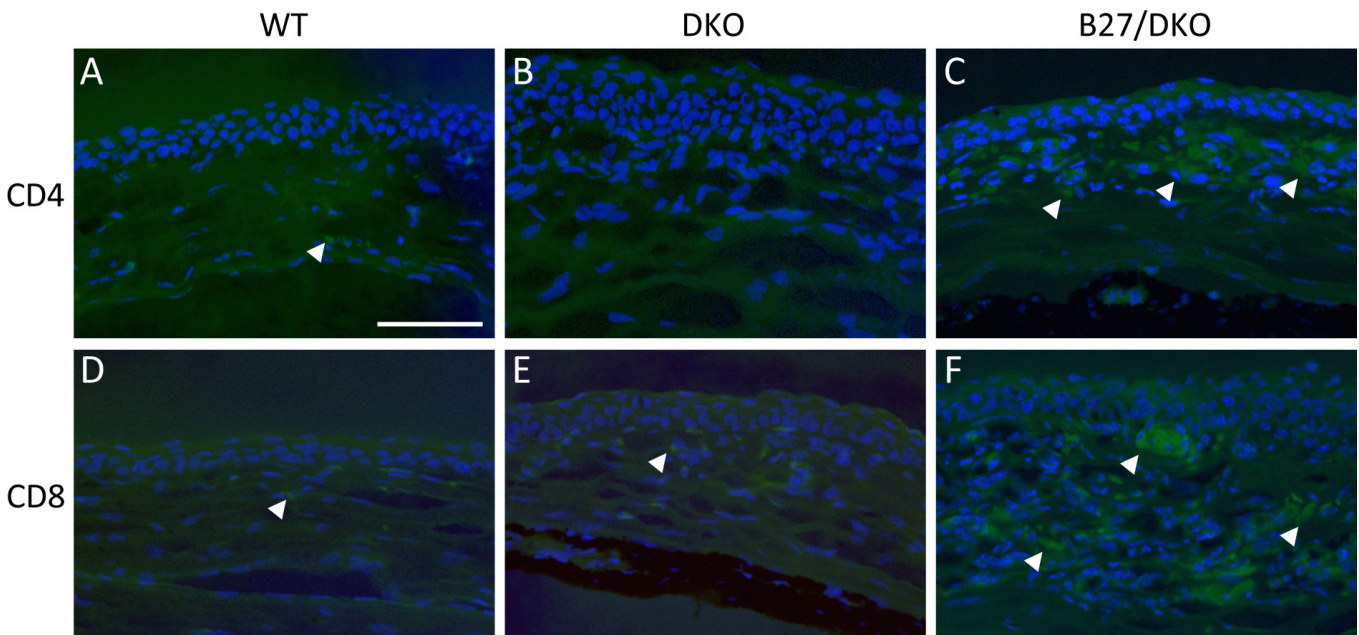


Figure 5. Persistent CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltrates in HLA-B27/DKO corneas. Central corneas from debrided animals at day 14 were stained with antibodies raised against CD4 and CD8. WT (**A, D**) and DKO (**B, E**) eyes were largely negative at this time, but HLA-B27/DKO corneas (**C, F**) retained strongly positive cells for both markers (arrowheads). (n = 4 animals, bar indicates 50 μM).

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