

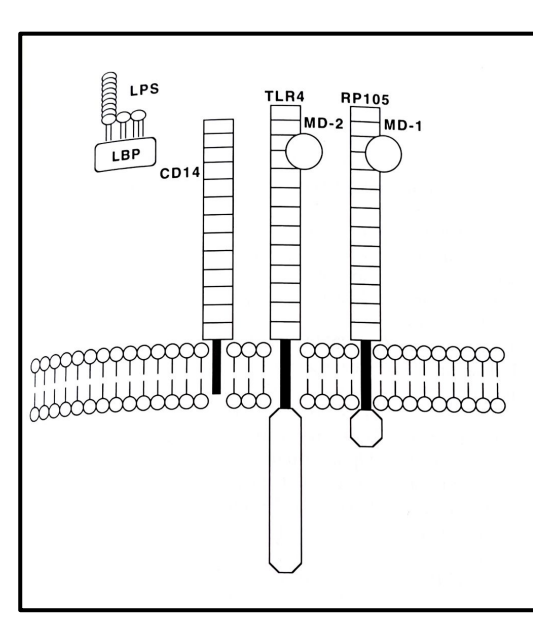
# The Effects of $\alpha$ -MSH and NPY on CD14 Production by Phagocytizing Macrophages

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## Introduction

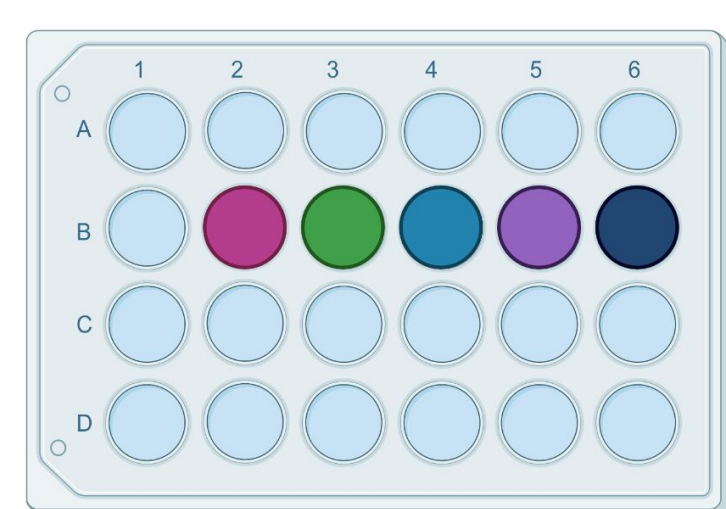
Upon recognition of lipopolysaccharide (LPS), a component of gram-negative bacteria, CD14 works alongside various TLR signaling complexes in order to induce an intracellular cascade. This leads to the secretion of proinflammatory cytokines and the subsequent activation of the innate immune system. As one of the few immune privileged areas within the body, the eye has adopted a series of mechanisms that suppress the traditional immune response to preserve vision. This is accomplished in part through retinal pigment epithelial cell (RPE) production of neuropeptides, which modulate the recruitment and activation of macrophages. Two of these neuropeptides, Neuropeptide Y (NPY) and alpha-Melanocyte Stimulating Hormone ( $\alpha$ -MSH), have been found to induce the secretion of anti-inflammatory cytokines and reduce the phagocytic activity of stimulated macrophages. In this study, we examined whether NPY or  $\alpha$ -MSH altered the production of the TLR-accessory molecule CD14 to further understand their role in regulating the inflammatory activity of phagocytizing macrophages.



**Figure 1: LPS receptor complex**  
LBP is a soluble molecule that binds to the lipid A portion of LPS, forming a complex that then binds to CD14. Because CD14 lacks a cytoplasmic region, it is unable to transduce a signal from the membrane into the myeloid cell on which it rests. As a result, the LBP-LPS-CD14 complex associates with TLR4, which then becomes responsible for initiating a signal transduction pathway.

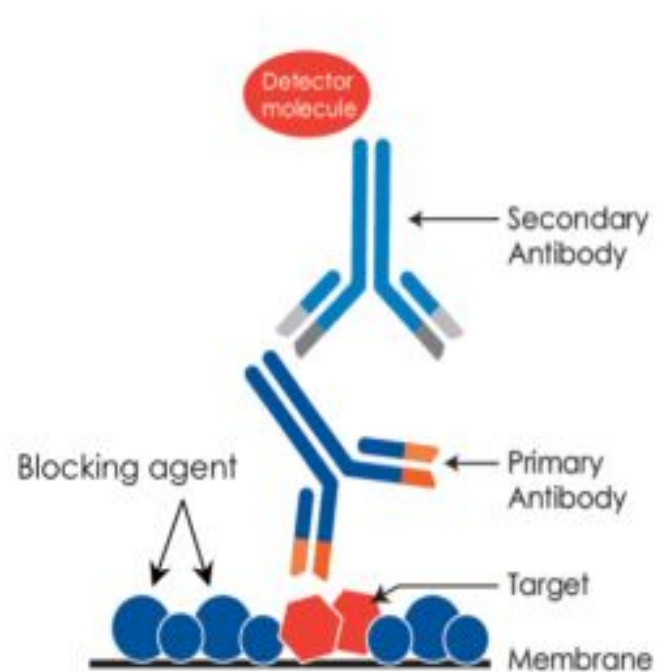
## Methods

A. The macrophage cell line, RAW cells, were added into 5 wells within a 24-well plate. The groups were then treated with the corresponding neuropeptides and activated by phagocytizing bioparticles.



**Well-plate map**  
This well-plate map is a pictorial representation of the five groups that comprised the experiment: media only (negative control), untreated, NPY-treated,  $\alpha$ -MSH-treated, and  $\alpha$ -MSH/NPY-treated macrophages.

B. Following a 24-hours of incubation at 37°C in 5% CO<sub>2</sub>, the macrophages were collected and lysed. The protein concentration of the lysate was measured, and the lysate was applied to a NuPAGE gel for gel electrophoresis. Once the gel electrophoresis was completed, the gel was transferred to a membrane for Western blotting. The membranes were blotted with antibodies to CD14 and to the loading control protein  $\beta$ -actin. The blotting was imaged, and the band intensities were measured. The relative expression of CD14 protein in the cells was then calculated for analysis.



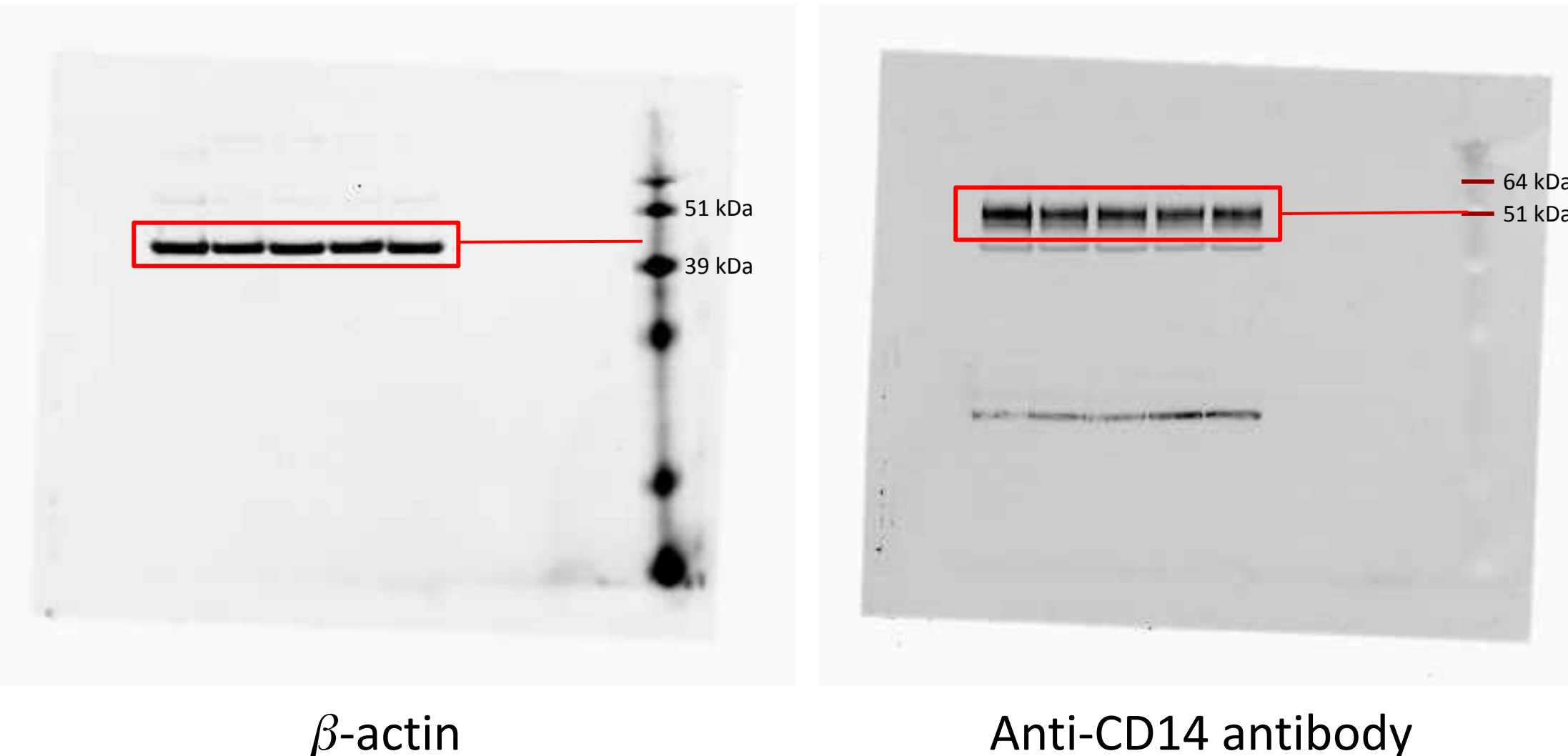
**Western blot procedure**  
Prior to the addition of the antibodies, the membrane was soaked in blocking buffer to prevent the antibodies from binding nonspecifically. The buffer was removed, and the primary antibody was introduced to bind to the target protein. The secondary antibody, which has the fluorescent marker attached to it, was then added to bind to the primary antibody, ensuring the results were capable of being detected.

C. In addition, an ELISA was run to measure the amount of soluble CD14 in the culture supernatant that was collected before the cells were gathered for lysis.

## Results

### Western Blot:

Figure 1: Western blot images



The band intensities were measured using imaging software. The band intensity for CD14 was divided by the band intensity for the respective  $\beta$ -actin band to assess the relative levels of CD14 present within each of the five experimental groups. This was calculated as follows.

Figure 2: Sample lane reports

Line 1	Name	#1
Line Information:		
Method	Radius	Sensitivity
Disk	2	1.0
	7.4	7.4
	0.0	0.0
	4.0	4.0
Band #	Band Name	Peak Int
1-1	#1	44272.00
1-2	#1	41001.00

### Step 1: Obtaining protein levels

$$\frac{\text{GT CD14}}{\text{GT } \beta\text{-actin}} = \frac{\#}{\text{Total protein mg}} = x/\text{mg}$$

Line 1	Name	#1
Line Information:		
Method	Radius	Sensitivity
Disk	2	1.2
	7.4	7.4
	0.0	0.0
	4.0	4.0
Band #	Band Name	Peak Int
1-1	#1	10572.00

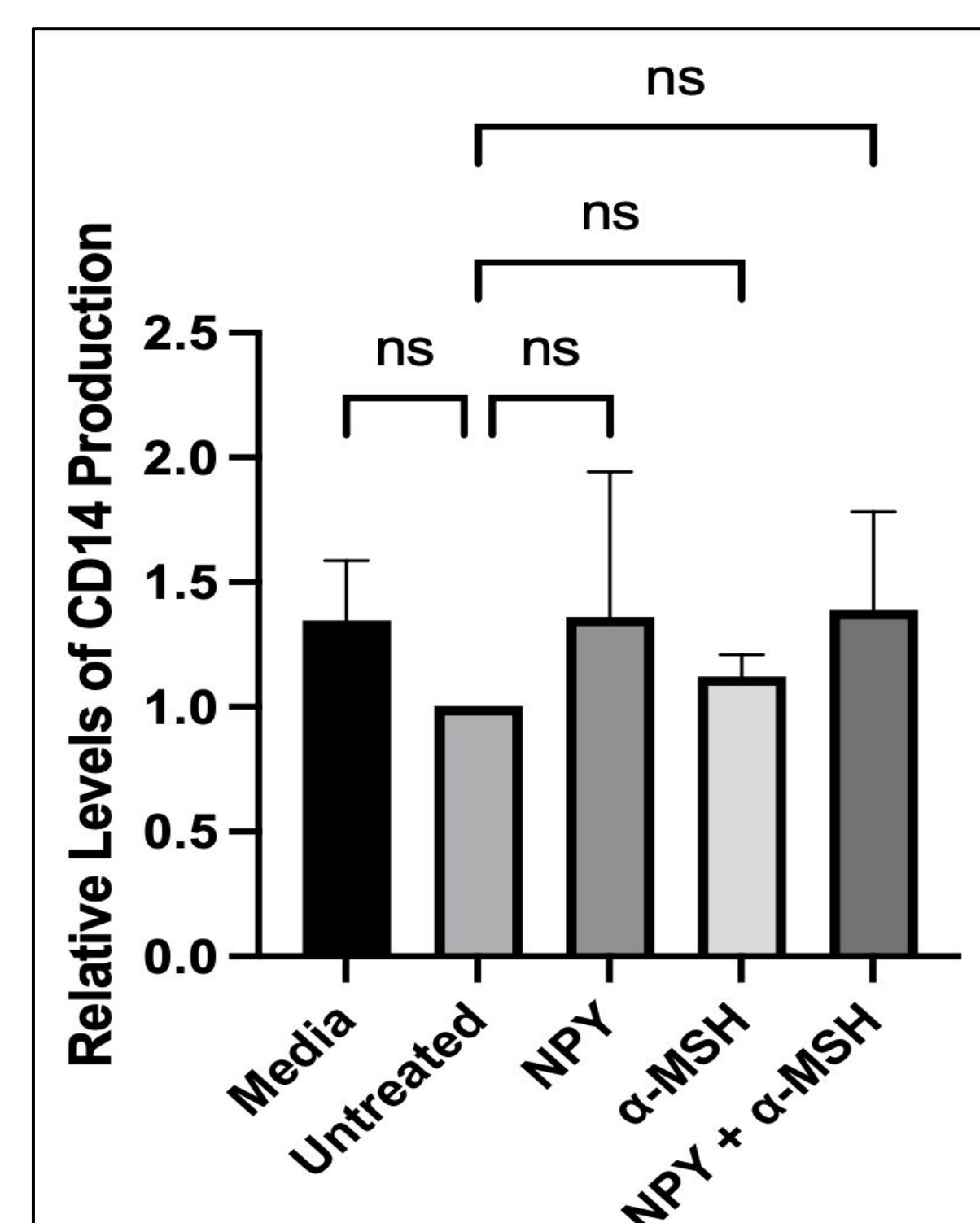
### Step 2: Transforming into relative protein levels

By comparing each result to that of the untreated group, our positive control, a ratio was gathered.

$$\frac{(x/\text{mg}) \text{ test}}{(x/\text{mg}) \text{ pos.}} = \text{relative ratio}$$

Four sets of ratios were obtained throughout the duration of the experiment. Despite the appearance of certain subtle trends, the results demonstrated that there was no statistical difference between the groups. This indicates that the neuropeptides had no effect on the production of CD14.

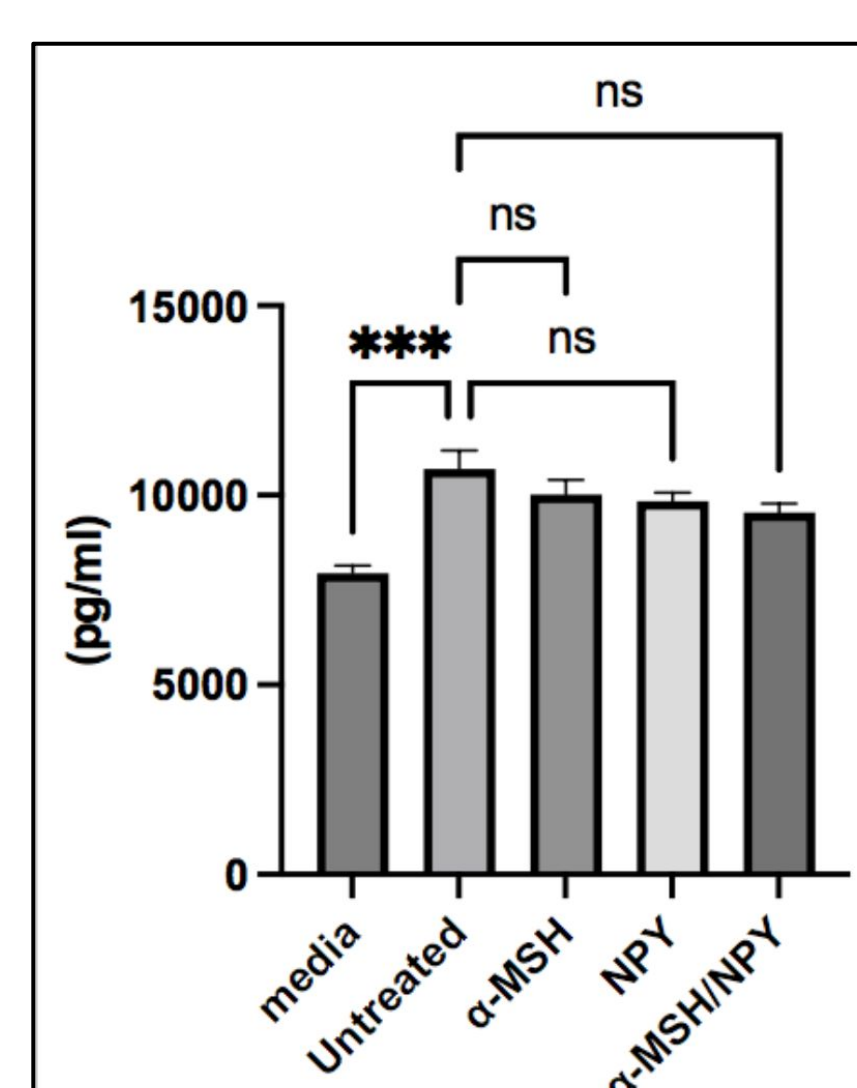
Figure 3: Relative expression of CD14



### ELISA for soluble CD14:

The results showed no statistical difference between the treated groups; however, it is clear that adding bioparticles for phagocytosis significantly increased the release of soluble CD14.

Figure 4: Concentration of soluble CD14



## Discussion/Conclusions

The results from both analyses indicated that NPY and  $\alpha$ -MSH neither alter CD14 production, nor do they change the amount of soluble CD14 being released by the activated macrophages. This suggests that the suppression of TLR-signaling by the neuropeptides does not occur through the suppression of the TLR-accessory molecule CD14.

In addition to its role within the inflammatory response, it has been found that the presence of CD14 allows phagocytes to clear dead and damaged cells and materials within a healthy eye. Because the neuropeptides suppress toll-like receptor proinflammatory signals within phagocytes, this would occur without an inflammatory response. As a result of this phagocytic process continuing without inflammation, it would allow for the maintenance of ocular immune privilege and the preservation of vision.

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