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Development of an improved *ex vivo* model of corneal inflammation and wounding

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Abstract

Purpose- The aim of this work was to develop an *in vitro* model of the cornea to study the effect of inflammation on wound healing.

Methods- Initial studies investigated how to maintain the *ex vivo* models for up to 4 weeks without loss of epithelium. To induce inflammation, corneas were cultured with interleukin (IL) 17A, IL 22, a combination of IL-17A and 22 or lipopolysaccharide. The effect of inflammation on wound healing was then examined.

Results- With static culture conditions, organ cultures deteriorated within 2 weeks. With gentle rocking of media over the corneas and carbon dioxide perfusion the *ex vivo* models survived for up to 4 weeks without loss of epithelium. The cytokine that caused the most damage to the cornea was IL-17A. Results showed that under static conditions wound healing of the central corneal epithelium occurred within 9 days with only a single layered epithelium formed whether the cornea was exposed to IL-17A or not. On rocking media gently over the corneas, a multilayered epithelium was achieved 9 days after wounding. In the presence of IL-17A however, there was no wound healing evident. Characterisation of the cells showed that wherever epithelium was present both differentiated cells and highly proliferative cells were present.

Conclusions- We propose that introducing rocking to extend the effective working life of this model and the introduction of IL-17A to this model to induce aspects of inflammation extend its usefulness to study the effects of agents which influence corneal regeneration under normal and inflamed conditions.

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Introduction

The cornea, through which light enters the eye, is a transparent, avascular tissue [1] which is responsible for keeping bacteria from entering into the eye [2] while at the same time allowing oxygen to enter [3]. This is achieved through tight junctions present at the superficial layer of the cornea creating a barrier [4]. This barrier function if violated makes the cornea and possibly the whole eye susceptible to infection [5].

Physical damage to the corneal epithelium is the most common cause of wounding. Wound healing of the cornea involves the removal of necrotic cells, infiltration of neutrophils and migration of cells from the wound edge covering the wound along with simultaneous multilayer formation [6].

Corneal injuries are usually associated with some level of inflammation which includes neutrophils [7], macrophages, dendritic cells and lymphocytes [8]. Neutrophils are recruited from the limbal blood vessels [7] and tear fluid [6] to the injury site just after stromal apoptosis takes place [9] due to IL-1 [10] and TNF-α [11] released by the injured epithelial cells. The neutrophils phagocytose cellular debris and pathogens [12, 13] clearing up the site of injury and also release large amounts of cytotoxic agents which assist in the phagocytosis of microbes [14].

Most cornea organ culture models employed to study the transfer of cells to the cornea or the effects of chemicals on the corneal epithelium and wound healing [15-19] have a life span of around 3-4 weeks at maximum and most of these models are static cultures [15, 16, 20].

The static models while fairly simple to set up, do not mimic the situation *in vivo* where the corneas are kept intermittently moist through the blinking action of the eyelids but they are not submerged. In the current study the corneas were placed in a simple rocking system (based on a commercially available egg incubator modified for CO_2

perfusion). Using this technique, multiple corneas could be cultured and we demonstrate that the models last for much longer than under static conditions.

We then used these to develop an *in vitro* cornea wound model to which cytokines were applied to induce inflammation. We identified a cytokine to induce aspects of inflammation and studied wound healing in the model over 9 days under both static and rocking culture conditions. We show that rocking provides a model in which wound healing occurs rapidly except when in the presence of the cytokine IL-17A.

Materials and methods

Cornea organ culture

New Zealand white rabbits were obtained from a rabbit farm in Hampshire, UK. The rabbits weighed between 2.4-2.6 kilograms. Excess fat and tissue was excised from the eyes and then were processed as described previously [16]. The corneo-scleral buttons were cultured at an air-liquid interface in 1 ml of medium consisting of Dulbecco's Modified Eagle Medium (DMEM GlutaMAX™, Gibco Life Technologies Ltd., Paisley UK) and Ham's F12 medium (Biosera, Ringmer, UK) in a 1:1 ratio supplemented with 10% foetal calf serum (Biosera, Ringer, UK), 10 ng/ml EGF, 5 µg/ml insulin, 2.5 µg/ml Amphotericin B and 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma Aldrich, Poole, UK). The corneas were cultured at 37˚C in a humidified, carbon dioxide (CO_2) environment. The medium of the corneas were changed every other day and kept wet by dropping media on the surface every day.

Corneal inflammation induction

On day 7, the medium of the unwounded corneas were changed to contain 20 ng/ml of recombinant human IL 17A (Peprotech, London, UK), 25 ng/ml of IL 22 (Peprotech, UK), a combination of IL-17A and 22 (20 ng/ml and 25 ng/ml respectively) or 1 µg/ml of lipopolysaccharide (LPS). The medium was changed every other day with wetting the corneas every day. On day 14, the samples were fixed using 3.7% buffered formaldehyde and processed for conventional histology.

Rocking culture of the corneas

After preparation of the corneo-scleral button, corneas were placed at the periphery of a 90mm petri dish and held in place by the end of a pipette tip glued onto the petri dish using araldite® epoxy adhesive (Araldite, Switzerland). The corneas were cultured in 13 mls of culture medium and placed in our *in-house* rocking system consisting of an egg incubator (R-COM King Suro Egg Incubator, P&T Poultry, Powys, UK) which was subsequently placed on a platform rocker (Stuart, Platform Rocker STR6, Stone, UK (Fig. 1) set at a speed of 10 rpm and maximum tilt angle of 6 degrees. The corneas were maintained at 37˚C in the humidified chamber of the egg incubator (Fig. 1). Sixteen corneas could be handled in the incubator at one time.

Assessment of the effect of including a CO_2 supply in the *in-house* rocking incubator

Cornea organ cultures were cultured in the *in-house* rocking incubator in the absence and presence of 5% CO_2. CO_2 was added through a hole at the top of the egg incubator via tubing from a cylinder containing 5% CO_2. The corneas were cultured for either two

or four weeks and compared to corneas cultured under static conditions in a conventional cell incubator (in the presence of CO_2) with the media changed every week.

Corneal epithelial wounding

After cleaning the eyes of excess tissue, the corneas were held in position and the central area of the cornea was wounded using 20% methanol within an 8 mm diameter trephine. After 40 seconds the area was rinsed with PBS to remove any excess methanol and using a sclerotome knife the epithelium was scraped off from the treated area (Fig. 2). The corneo-scleral buttons were excised and then processed to be placed in 90 mm petri dishes as described previously. Organ cultures with unwounded epithelia were also set up as controls.

On day 2, the media of the wounded and unwounded corneas were changed to contain the cytokine that resulted in inflammation (Fig. 2). Controls were wounded and unwounded corneas with the organ culture medium and no cytokine. On day 7 the corneas on the rocker were transferred to 35 mm petri dishes containing 1 ml of medium with cytokines and on day 9 the corneas were fixed using 3.7% buffered formaldehyde and processed for conventional histology. This rocking protocol was compared to a previous protocol employed [16] placing the corneas in a 35 mm petri-dish from the start in an incubator maintained at 37˚C in a humidified, 5% CO_2 environment under static conditions.

144 **Immunohistochemistry**

145 Corneal sections of 4 µm were mounted onto Superfrost® Plus microscopic slides
146 (Thermo Fisher Scientific, Loughborough, UK) after which they stained for cytokeratin 3
147 (CK3) and P63 as described previously [16]. The samples were imaged using a Zeiss LSM
148 510 META confocal microscope at laser wavelength 488 nm and 543 nm at the Kroto
149 Research Institute Confocal imaging facility.

150

151 **Results**

152 **Investigation of the effect of culturing corneas under rocking versus static**
153 **conditions**

154 We assessed the integrity of the corneal epithelium under static and rocking conditions.
155 In order to mimic the blinking action of the eye lids and consequently keep the corneas
156 wet, the corneas were placed on a rocker within an incubator maintained at 37˚C with a
157 small volume of media which flowed intermittently over the cornea by the rocking action.
158 These results were compared to corneas cultured within static incubators at 37˚C.

159 Figure 3 shows the effect of CO_2 on the corneas cultured under rocking conditions. It
160 could be clearly seen that culturing the corneas under static conditions in the presence
161 of CO_2 for 2 weeks (Fig. 3a) and 4 weeks (Fig. 3d) resulted in a 1-2 cell layer thick
162 epithelial while under rocking conditions without (Fig. 3b) and with (Fig. 3c) CO_2 after 2
163 weeks resulted in a 5 cell layers thick epithelium. On culturing the corneas under rocking
164 conditions for 4 weeks, the epithelium was completely lost in the absence of CO_2 (Fig.
165 3e) but was retained in the presence (Fig. 3f). After 2 weeks it was observed that the
166 organisation of the epithelium was poor in the absence of CO_2 (Fig. 3b) compared to the
167 corneas cultured in the presence of CO_2 (Fig. 3c) and after 4 weeks even in the

presence of CO_2 (Fig. 3f) the epithelium appeared to have lost the superficial desquamated cells compared to after 2 weeks in culture (Fig. 3c).

Effect of inflammatory cytokines on corneal epithelium

Previous studies [21] and unpublished studies in the group have shown that exposure to cytokines IL-17A and IL-22 leads to inflammation of the epidermis and a combination of IL-17A and 22 further enhanced inflammation of human colonic subepithelial myofibroblasts [22]. Similarly LPS has been shown to induce inflammation of the epithelium [23, 24]. Using these cytokines and LPS, the inflammation of the corneal epithelium was studied.

On application of the cytokines/LPS on day 0 in culture, there did not appear to be any changes in the epithelium even after one week in culture (images not shown). The corneas were then cultured for 7 days prior to the application of the cytokines as there may not have been enough time for the corneal barrier function to break down *in vitro* to allow their entry.

Figure 4 shows that using IL-17A (Fig. 4b) and a mixture of IL-17A and 22 (Fig. 4d) resulted in a poor epithelium with a very loosely attached multilayer of cells. Addition of IL-17A led to a split in the epithelial multilayer. The basal cells remained well adhered to the corneal surface with a suprabasal split of the cells above this. The control cornea which had not been exposed to the cytokines showed a normal multilayer of cells on the corneal surface (Figure 4a) and similar epithelia to the control was seen on corneas exposed to IL-22 (Fig. 4c) and LPS (Fig. 4e).

Effect of inflammation on wound healing

In this study the corneas were cultured for 2 days prior to the application of the cytokine to induce inflammation. Figure 5 shows the effect of inflammation on wound healing of the cornea. Fig. 5a shows a normal cornea under static conditions. Here there was no multilayer formation and after 9 days in culture there was just a single layer of cells on the corneal surface. The effect of IL-17A (Fig. 5b) on the unwounded cornea resulted in again a single layer of cells on the surface with the cells appearing abnormal with some level of perinuclear vacuolation. The wounded cornea (Fig. 5c) was about one layer thick and a similar epithelium was seen when the wounded cornea was exposed to IL-17A (Fig. 5d).

Although the images show the central area of the cornea where wounding was carried out, the epithelium shown in the images is a representation of the whole corneal epithelium.

Under rocking conditions however, the control unwounded cornea had a much improved epithelium compared to the cornea cultured under static conditions and had about 7 layers in thickness (Fig. 5e). IL-17A had an adverse effect on the unwounded cornea resulting in the loss of most of the epithelium and only a very few cells on the surface (Fig. 5f). Nine days after wounding the control cornea, a multilayered epithelium had formed of about 5 cell layers thick under rocking conditions (Fig. 5g) however on exposing the wounded cornea to IL-17A, the cornea lost all the epithelial cells and there was no regeneration of epithelium visible even under rocking conditions (Fig. 5h). Also in the wounded corneas it was noted that the stromal cells were not present in the area where wounding had taken place (Fig. 5c-h).

Immunohistochemistry showed that the cells on the corneal surface expressed CK3 despite being exposed to IL-17A under both static and rocking conditions (Fig. 6a-g).

P63 continued to be expressed in all the corneas whether exposed to IL-17A or not (Fig. 6i-o). However, this was more prominent at the peripheral area of the cornea than the central area. P63 could also be visualised in the basal layer of cells in the central corneal region where a multilayer of cells formed in the unwounded (Fig. 6m and fig. 7a-c) and wounded (Fig. 6o and fig. 7d-f) corneas under rocking conditions but could not be seen where only a monolayer of cells had formed (Fig. 6i-l, n). No CK3 or P63 expression was observed in the wounded corneas exposed to IL-17 under rocking conditions (Fig. 6h, p) as there were no cells present on the corneal surface as seen using H&E staining (Fig. 5).

Discussion

Corneal neovascularisation is known to be associated with inflammation of the cornea [25] and this may lead to the loss of the corneal immune privilege [26]. If a patient has a condition with associated inflammation and the treatment requires transplantation of tissue/cells, then the inflammation can have a direct impact on the transplanted tissue/cells and rejection may take place. The ingress of blood as well as lymphatic vessels caused by inflammation onto the avascular cornea leads to the movement of antigen presenting cells into the corneal region which may eventually lead to the rejection of donor tissue [27]. To support this, studies have shown that rejection of corneal graft transplantation is much higher on vascularised corneas than avascular corneas [27, 28]. Therefore there is a need to control the inflammation of the cornea before or while any transplantation or treatment takes place. This may be done by using anti inflammatory drugs [29] or using cell carriers with incorporated anti-inflammatory properties such as the amniotic membrane [30]. Our group has shown that Ibuprofen can

241 be incorporated into poly (lactide-co-glycolide) scaffolds [31]. As these scaffolds degrade
242 the anti-inflammatory drug is released similar to the amniotic membrane [32].

243 From the current study we suggest the use of 3D cornea models can be improved quite
244 simply by rocking the corneas from side to side in order to mimic the blinking action of
245 the eye. Blinking provides the cornea with fresh tears and nutrients to the corneal cells
246 [33]. Richard et al in 1991[19] placed corneas on a standard laboratory rocking platform
247 where the intermittent movement of fluid over the cornea was used to mimic the
248 exposure to tear fluid via blinking. They compared the rocking technique to corneas
249 cultured under static submerged conditions. Results showed that culturing the corneas
250 intermittently at an air-liquid interface reduced epithelia, stromal and endothelial
251 intercellular edema. However, the technique of placing the rocker in an incubator may
252 make the incubator system susceptible to overheating and this method does not seem to
253 have been taken up by others. In 2006 a more complex perfusion model [18] was
254 published in which media was delivered drop-wise on to a cornea to mimic the blinking
255 action instead.

256 In the current study the corneas were placed in a simple rocking system (based on a
257 commercially available egg incubator modified for CO_2 perfusion). Using this technique,
258 it was readily demonstrated that the 3D models lasted for much longer than under static
259 conditions.

260 With respect to culturing corneal limbal stem cells for transplantation, some groups have
261 cultured cells on an amniotic membrane at an air-liquid interface [34] to form a multilayer of
262 cells prior to transplantation while others have cultured the cells under submerged
263 conditions [35] forming only a monolayer of cells. It is interesting to note that both have
264 worked clinically. With the current technique it would be possible to place an amniotic
265 membrane or a synthetic membrane on the cornea and look at the formation of an

epithelium from cultured cells or indeed from explants of tissue under rocking conditions to see which provides best epithelial regeneration.

In order to establish the model, the effect of including a CO_2 supply in the rocking incubator was first studied. The presence of CO_2 was crucial for pH maintenance around 6.9-7.8 which is important for cells to grow [36]. In cultured cells, the bicarbonate leaves the medium in the form of carbon dioxide making the medium very alkaline. The presence of 5% CO_2 maintains an equilibrium in the tissue culture dish, preventing the increase in the pH to over 8.5 [37] which is toxic to the cells [38]. The results confirmed the necessity of a CO_2 supply to our in-house incubator, especially while culturing these corneas for long periods of time (4 weeks) compared to shorter periods (2 weeks).

T helper cells play a vital role in immune response [39] and these can be divided into Th1, Th2 and Th17 [40]. Th-17 is a T helper cell subset which on differentiation releases IL-17, 21 and 22 with IL-1, IL-6 and TGFβ1 playing a major role in driving the differentiation process [41] while IL-22 is also sourced from Th2 which play a role in immunity and remodelling [42]. IL-17A, a member of the IL-17 cytokine family is known to play a role in host defence against pathogens in both mucosal and epithelial tissue and IL-22, a member of the IL-10 cytokine family plays a pivotal role in epithelial wound healing [43, 44].

IL-17 and -22 are known to drive both neutrophil infiltration as well as metalloproteinases (MMPs) [45]. Studies have shown that IL-17 in particular plays a role in disrupting the corneal barrier function due to the MMPs produced. This can have a large effect on the epithelial cell multilayer causing erosion and surface irregularity [46]. Studies have also shown that IL-17RA (the receptor of IL-17A) is highly expressed in the basal limbal region suggesting that the region is susceptible to the inflammatory action of IL-17 [47]. Donetti et al reported that IL-17 reduces proliferation [48], is destructive to tissue and is labelled as a pro-inflammatory[42]. IL-22 has been reported to be responsible for the

291 inhibition of differential gene expression while inducing proliferation and migration of
292 keratinocytes [49]. IL-17 alone has been implicated in dry eye [46], scleritis, experimental
293 autoimmune uveoretinitis [50], herpetic stromal keratitis [51] and multiple sclerosis [52] while
294 IL-17 and IL-22 together have been implicated in diseases related to skin inflammation,
295 rheumatoid arthritis [52-54] and uveitis [50] This may explain the results in this study (Fig. 4)
296 where IL-17 or the combination of IL-17 and 22 affected the epithelial morphology on the
297 corneal surface.

298 In this particular study LPS, which is found on the cell wall of gram negative bacteria and
299 is an endotoxin known to cause inflammatory responses to the host tissue [55, 56], had little
300 or no effect on the appearance of the corneal model. Studies have shown that LPS may
301 cause an inflammatory response only if there is an entry site into the cornea but certainly
302 in these studies we failed to find any convincing response to LPS.

303 The major finding of this study was the extent to which subjecting corneas to gentle
304 rocking improved the maintenance of the epithelium. Not only did they survive longer (4
305 weeks under these conditions) than under static conditions but there was a clear benefit
306 when a wound was made in the model and it was allowed to recover. Recovery from
307 wounding was very effective when corneas were kept under rocking conditions, much
308 less so under static conditions. For the maturation of most 3D epithelial tissues *in vitro*,
309 exposure to an air-liquid interface is essential, as is the case for skin, buccal mucosa
310 and oesophageal mucosa [57-59]. However in the case of the cornea it has not been clear
311 whether for *in vitro* experimentation it is best to keep these submerged or at an air-liquid
312 interface or, as in this study, subject to intermittent bathing with media. This study clearly
313 shows the benefit of subjecting these epithelia to intermittent movement of media.

314 When epithelia are injured one of the first responses of the stromal cells is apoptosis of
315 keratocytes which can carry on for 1 week after the injury has taken place [60]. It was

noted in this study that most of the stromal cells close to the epithelial surface had died giving a clear indication of the area where the wounding had taken place on the cornea during these experiments as shown in Fig. 5.

With respect to expression of CK3 and P63, CK3 is known to be associated with the differentiated cells of the central corneal region [61] while Pellegrini et al [62] suggested that P63 is a stem cell marker expressed only in the basal layers of the limbal region. In characterisation studies it was seen that wherever cells were present on the corneal epithelium they expressed CK3 towards the central region of the cornea while P63 was expressed at the periphery which contains the limbus as well as the basal region of the corneal epithelium (fig. 7) which has also been reported previously.

We suggest that this relatively simple *ex vivo* rabbit cornea model can now more usefully be used to look at some of the drugs and cell delivery systems currently tested in live rabbit eyes.

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Figure legends

Figure 1. Photograph of a commercially available egg incubator on a commercially available platform rocker (A) and schematic of the cornea organ culture in a petri dish at a (i) horizontal position and (ii) inclined position on a platform rocker (B).

Figure 2. Schematic of an inflamed *in vitro* cornea wounded model

Figure 3. H&E of cornea organ cultures at 2 weeks under static conditions (A) and rocking conditions without (B) and with (C) CO_2 and at 4 weeks under static conditions (D) and rocking conditions without (E) and with (F) CO_2.

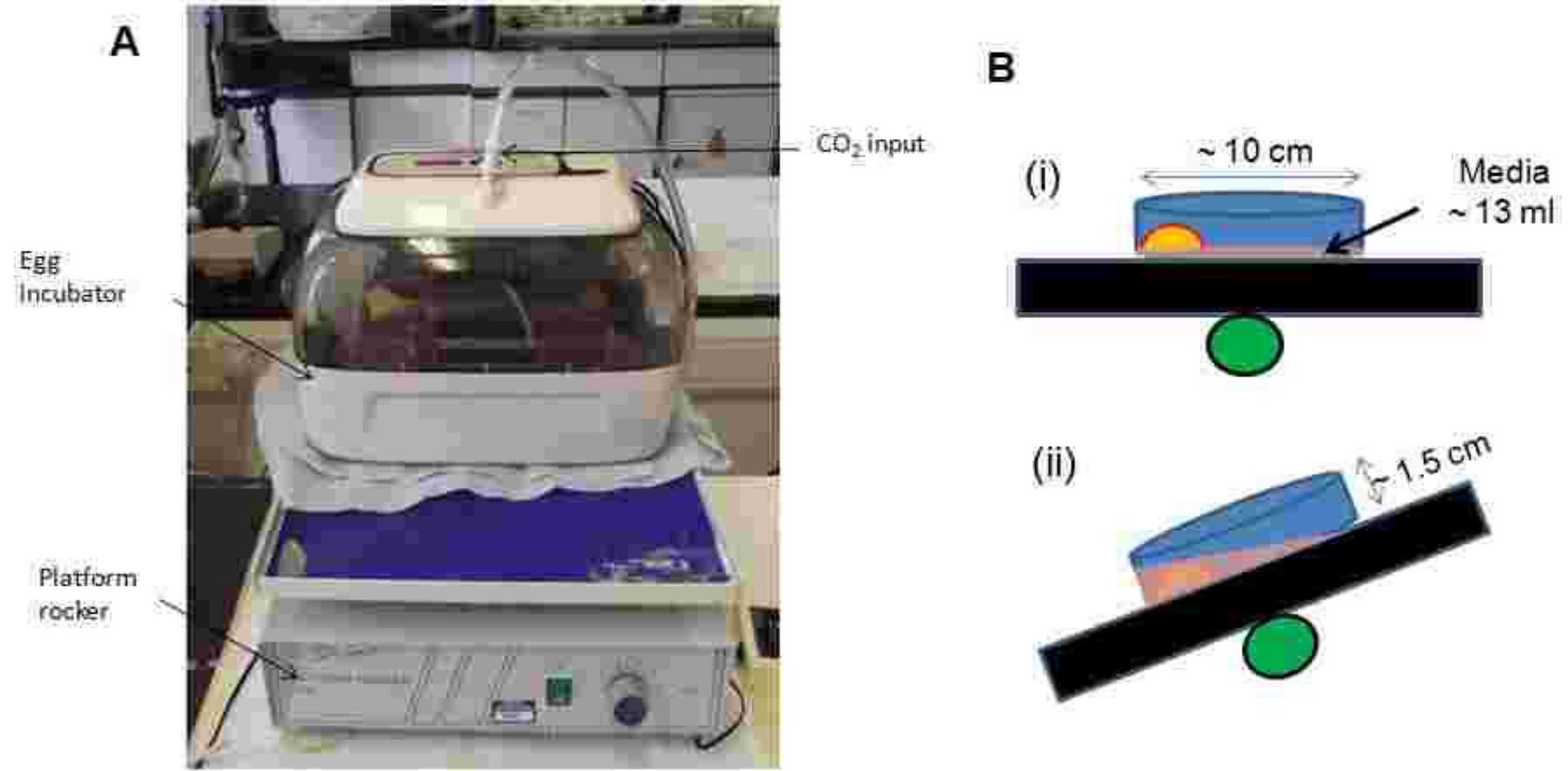
Figure 4. H&E of cornea organ culture exposed to cytokines. (A) shows the control cornea without cytokine exposure, (B) was exposed to IL-17A (20ng/ml), (C) was exposed to IL-22 (25ng/ml), (D) was exposed to both IL-17A and 22 (20ng/ml and 25ng/ml respectively) and (E) was exposed to lipopolysaccharide (1µg/ml)

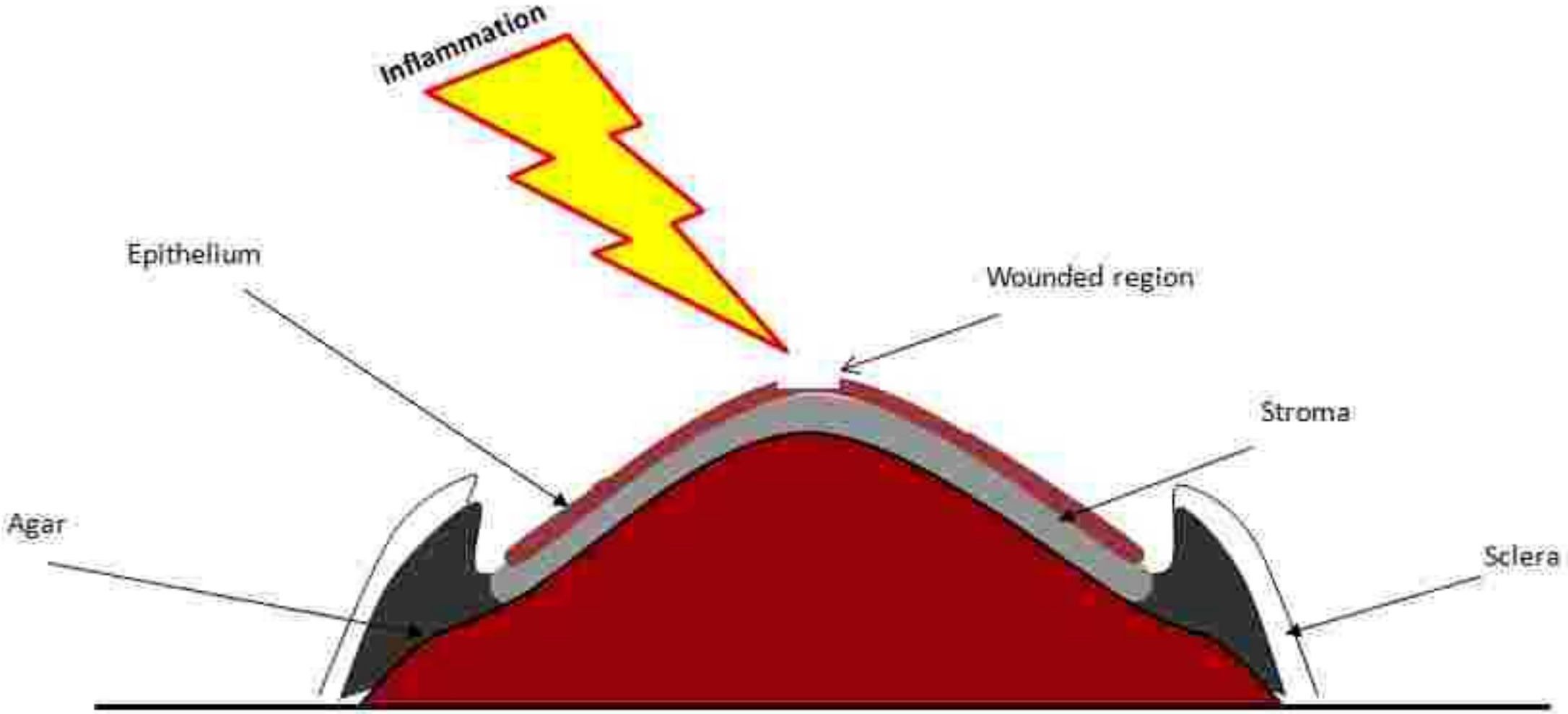
Figure 5. H&E of *in vitro* cornea organ culture after 9 days under static (A-D) and rocking (E-H) conditions. A,E shows unwounded corneas, B,F shows unwounded corneas exposed to IL-17A, C,G shows a wounded cornea and D,H shows a wounded cornea exposed to IL-17A.

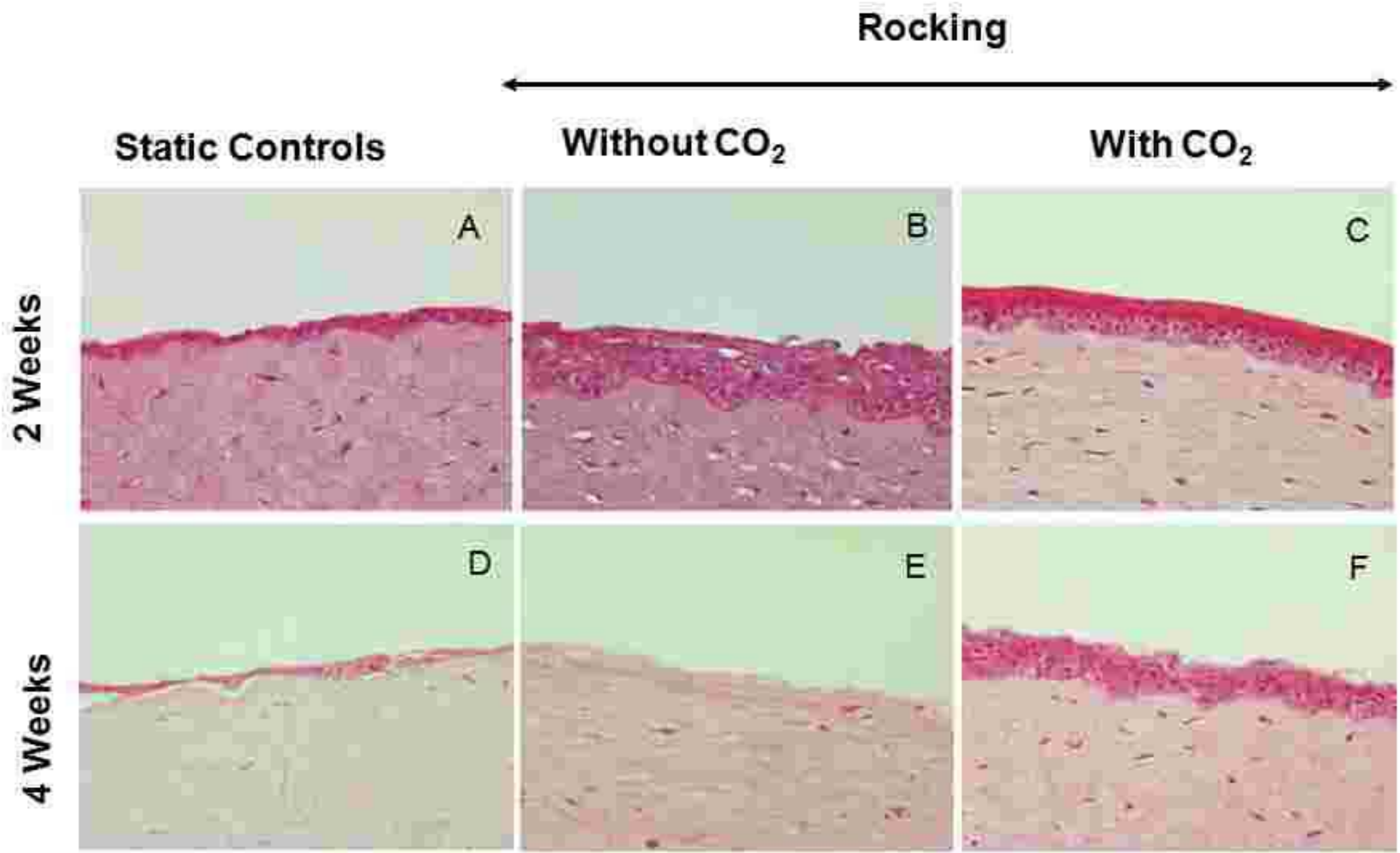
Figure 6. Expression of CK3 (A-H) and P63 (I-P) (shown in green) in cells on an *in vitro* corneal organ culture model under static (A-D and I-L) and rocking (E-H and M-P) conditions. A, E, I, M show an unwounded cornea (control), B, F, J, N show an unwounded cornea exposed to IL 17, C, G, K, O show a wounded cornea and D, H, L, P

529 show a wounded cornea exposed to IL 17. Nuclei were counterstained with propidium
530 iodide (red).

531 **Figure 7.** Expression of P63 in unwounded (A-C) and wounded (D-F) corneas cultured
532 under rocking conditions. Images show P63 (green) expressed in nuclei (indicated by
533 white arrows) in A and D, nuclei counterstained with propidium iodide (red) in B and E
534 and the merged image of the A and B and D and E in C and F respectively.


A
CO_2 input
Egg Incubator
Platform rocker
B
(i)
~ 10 cm
Media ~ 13 ml
(ii)
~ 1.5 cm

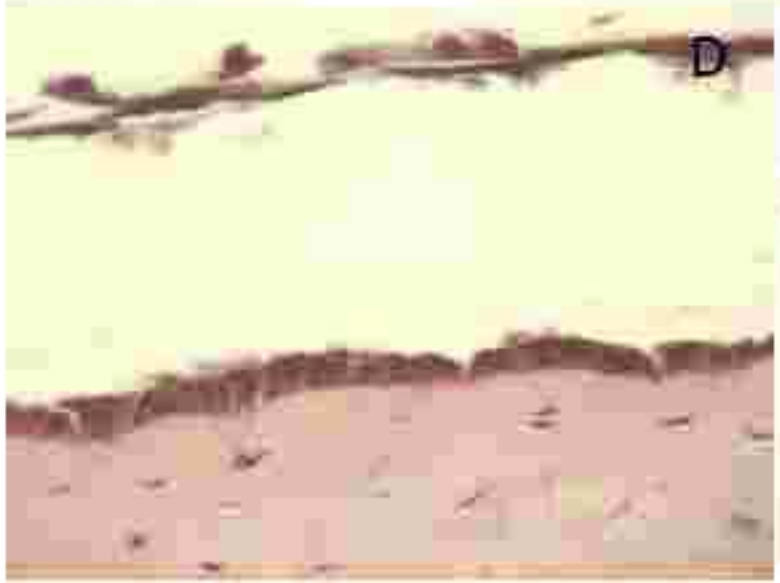

Inflammation
Epithelium
Wounded region
Stroma
Agar
Sclera


Rocking
Static Controls
Without CO_2
With CO_2
2 Weeks
4 Weeks
A
B
C
D
E
F

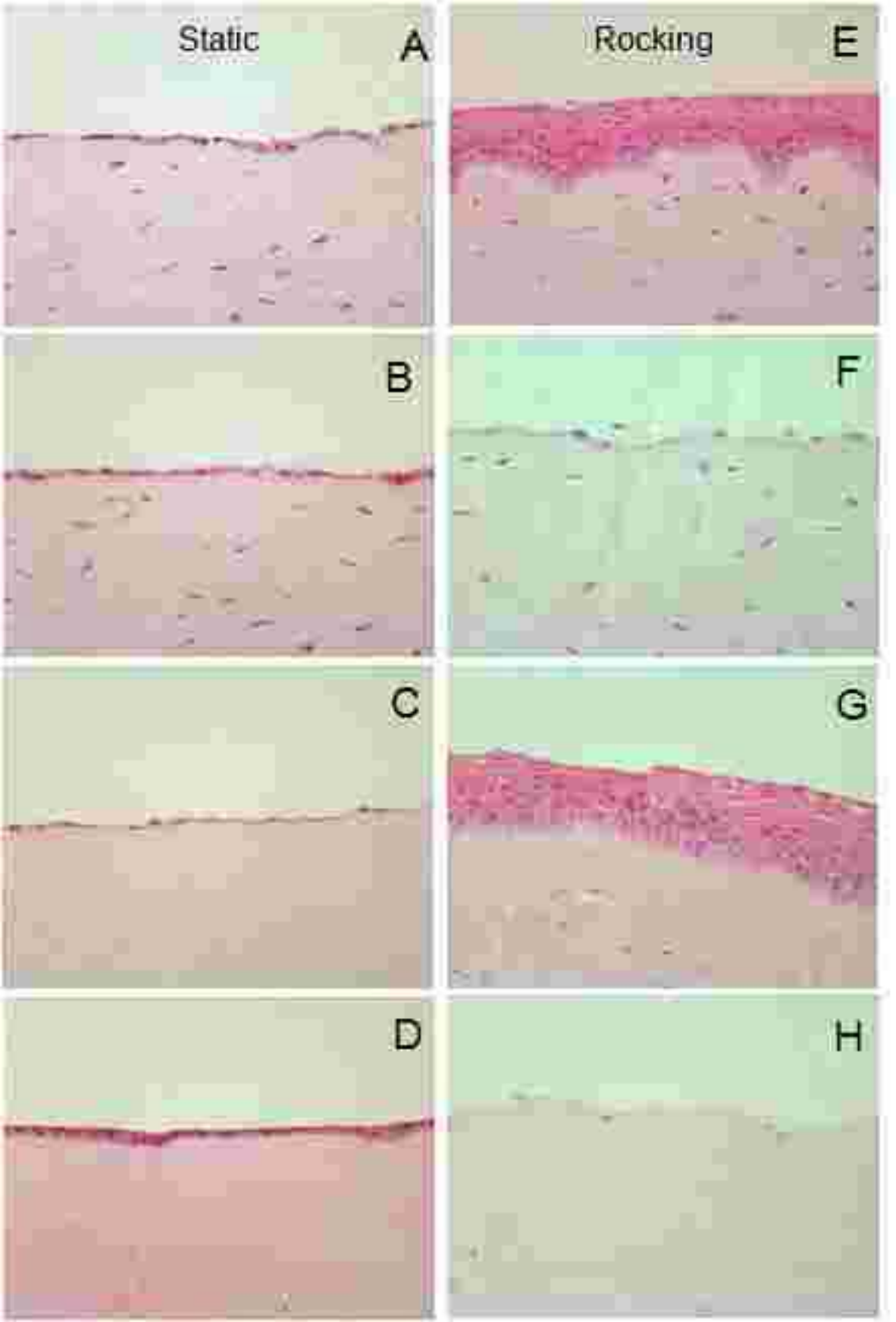

A

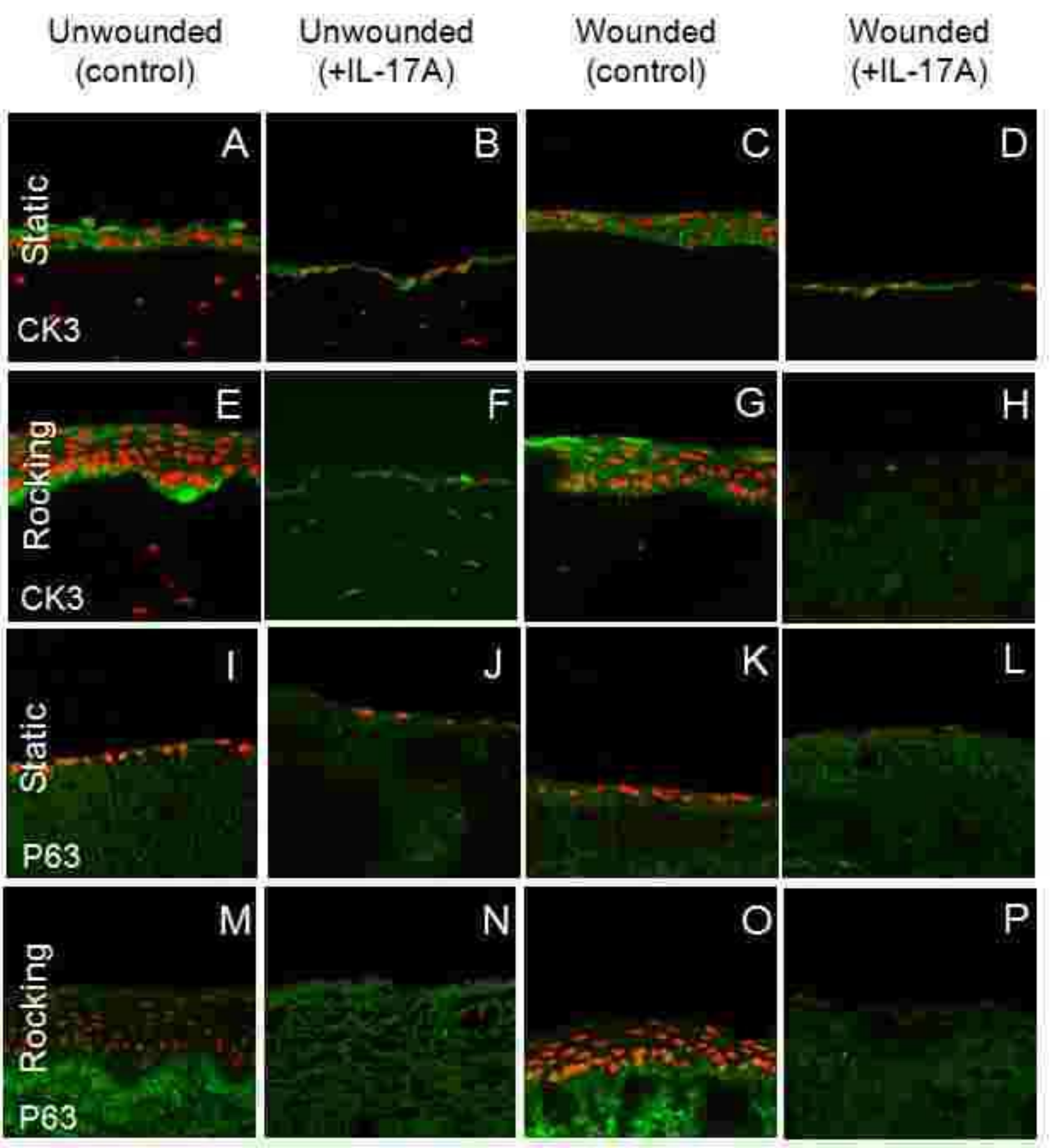

B

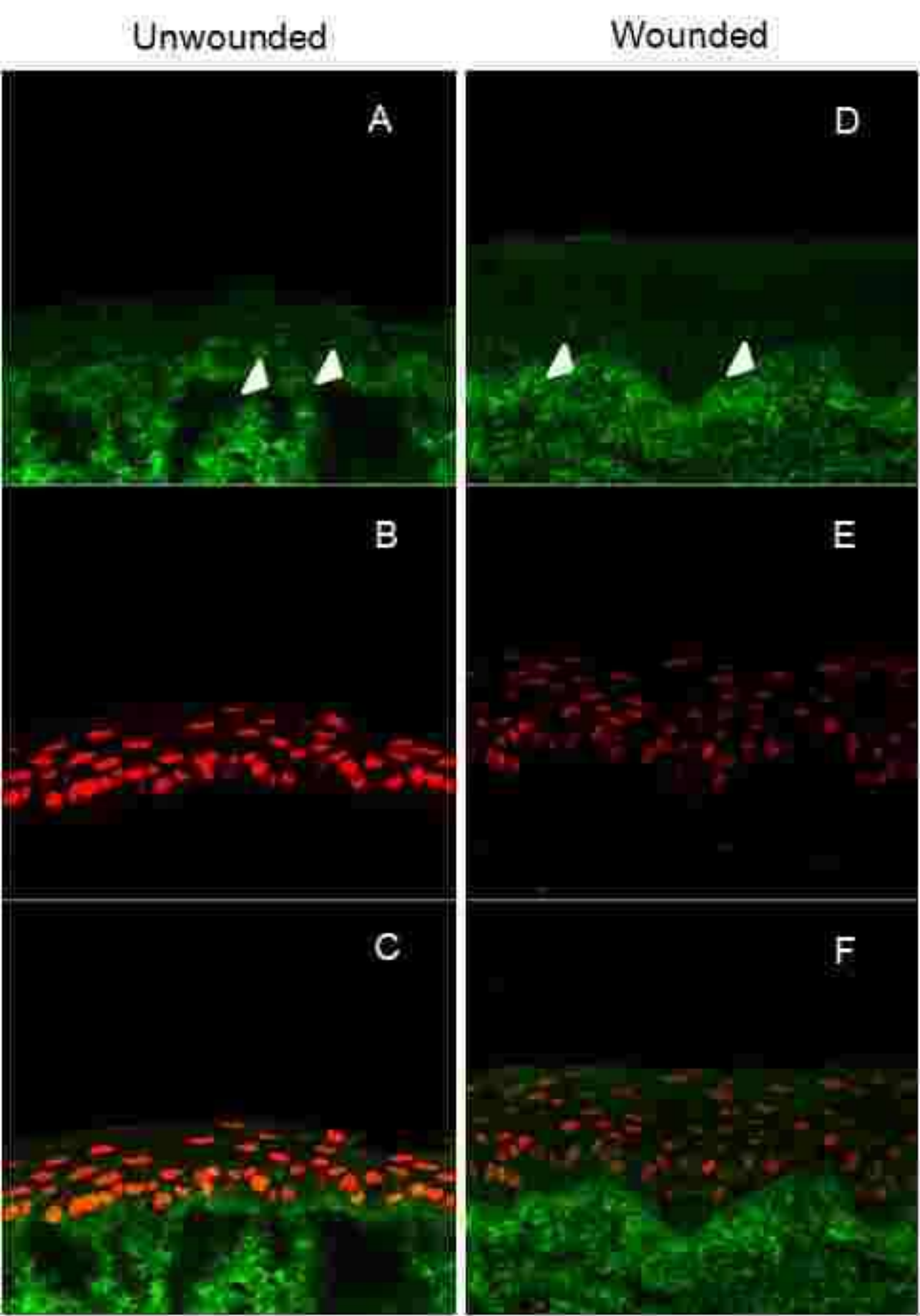

C


D


E


Static
A
Rocking
E
B
F
C
G
D
H


Unwounded (control)
Unwounded (+IL-17A)
Wounded (control)
Wounded (+IL-17A)
A
B
C
D
Static
CK3
E
F
G
H
Rocking
CK3
I
J
K
L
Static
P63
M
N
O
P
Rocking
P63


Unwounded
Wounded
A
D
B
E
C
F