Interaction of IFN-γ with cholinergic agonists to modulate rat and human goblet cell function

L García-Posadas[1,2], RR Hodges[3,4], D Li[3,4], MA Shatos[3,4], T Storr-Paulsen[3], Y Diebold[1,2] and DA Dartt[3,4]

Goblet cells populate wet-surfaced mucosa including the conjunctiva of the eye, intestine, and nose, among others. These cells function as part of the innate immune system by secreting high molecular weight mucins that interact with environmental constituents including pathogens, allergens, and particulate pollutants. Herein, we determined whether interferon gamma (IFN-γ), a Th1 cytokine increased in dry eye, alters goblet cell function. Goblet cells from rat and human conjunctiva were cultured. Changes in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), high molecular weight glycoconjugate secretion, and proliferation were measured after stimulation with IFN-γ with or without the cholinergic agonist carbachol. IFN-γ itself increased $[Ca^{2+}]_i$ in rat and human goblet cells and prevented the increase in $[Ca^{2+}]_i$ caused by carbachol. Carbachol prevented IFN-γ-mediated increase in $[Ca^{2+}]_i$. This cross-talk between IFN-γ and muscarinic receptors may be partially due to use of the same Ca^{2+}_i reservoirs, but also from interaction of signaling pathways proximal to the increase in $[Ca^{2+}]_i$. IFN-γ blocked carbachol-induced high molecular weight glycoconjugate secretion and reduced goblet cell proliferation. We conclude that increased levels of IFN-γ in dry eye disease could explain the lack of goblet cells and mucin deficiency typically found in this pathology. IFN-γ could also function similarly in respiratory and gastrointestinal tracts.

INTRODUCTION

The wet-surfaced mucosa including the conjunctiva of the eye, the intestine, colon, nose, bronchioles, Eustachian tube, and vagina contain goblet cells. These cells function as part of the innate immune system by secreting high molecular weight mucins that directly interact with environmental constituents including pathogens, allergens, and particulate pollutants. Substantial experimental evidence demonstrates that goblet cells function in mucosal epithelial protection and disease pathogenesis in respiratory and gastrointestinal tracts.[1,2]

In the ocular surface, goblet cells are found in the epithelial layer of the conjunctiva, the mucous membrane that surrounds the cornea, and lines the eyelids. These goblet cells are specialized cells that produce and secrete mucins, most notably the mucin (MUC) MUC5AC that lubricates and protects the ocular surface, maintaining its health.[3,4] Goblet cells are also integral participants in diseases of the ocular surface including allergic conjunctivitis, bacterial keratitis and conjunctivitis, and dry eye.

MUC5AC is a high molecular weight glycoconjugate that forms the mucous layer of the tear film.[5] The amount of MUC5AC found in the ocular surface is tightly controlled by goblet cell number, MUC5AC synthesis, and MUC5AC secretion. In inflammatory disorders such as dry eye, Sjögren's syndrome or ocular cicatricial pemphigoid goblet cells die or are non-functional.[6–8] On the other hand, in diseases such as allergic conjunctivitis, higher goblet cell numbers are found. As early as in 1992, Lemp[9] suggested that either an increase or a decrease in the number of filled goblet cells was associated with ocular surface pathology.

Under normal conditions, goblet cell secretion is under neural control by the efferent parasympathetic nervous system. Cholinergic, muscarinic mediators that are analogs of the parasympathetic neurotransmitter acetylcholine are the major stimuli.[10] Cholinergic agonists transmit their signal by activating the G protein $G_{\alpha q}/11$ that activates phospholipase C, which breaks down phosphatidylinositol 4,5 bisphosphate (PIP_2) producing inositol 1,4,5-trisphosphate (IP_3) and

diacylglycerol. The increase in IP_3 binds to its receptor in the endoplasmic reticulum to release Ca^{2+} from intracellular stores thereby elevating the intracellular Ca^{2+} concentration $([Ca^{2+}]_i)$.[11] The increase in $[Ca^{2+}]_i$ leads to activation of extracellular regulated kinase (ERK)1/2 (also known as p44, p42 mitogen activated protein kinase (MAPK)), and secretion of high molecular weight glycoconjugates including MUC5AC.[12] In airway epithelium, mucin secretion is dependent upon the calcium sensors Munc13-2 and synaptotagmin2.[13] These proteins have not yet been identified in the conjunctiva. Cholinergic agonists mediate goblet cell secretory responses to environmental changes under normal conditions. When inflammation develops in the ocular surface as occurs in

dry eye, these responses may be altered. This alteration would lead to a change in goblet cell mucin production. In early disease, mucus production can be increased as a protective response, but later in the disease, goblet cell mucin production can be decreased leading to ocular surface pathology.

Interferon gamma (IFN-γ) is the major Th1-derived cytokine. This cytokine is implicated in several different immune responses, such as inflammation or graft rejection. IFN-γ is secreted by cytotoxic T cells, Th1 cells, and natural killer cells.[14] It binds to its receptor, IFN-γ-R, that is ubiquitously expressed at the cell surface on all cells except erythrocytes.[15] The main signaling pathway induced by IFN-γ is through the JAK-STAT intracellular signal transduction
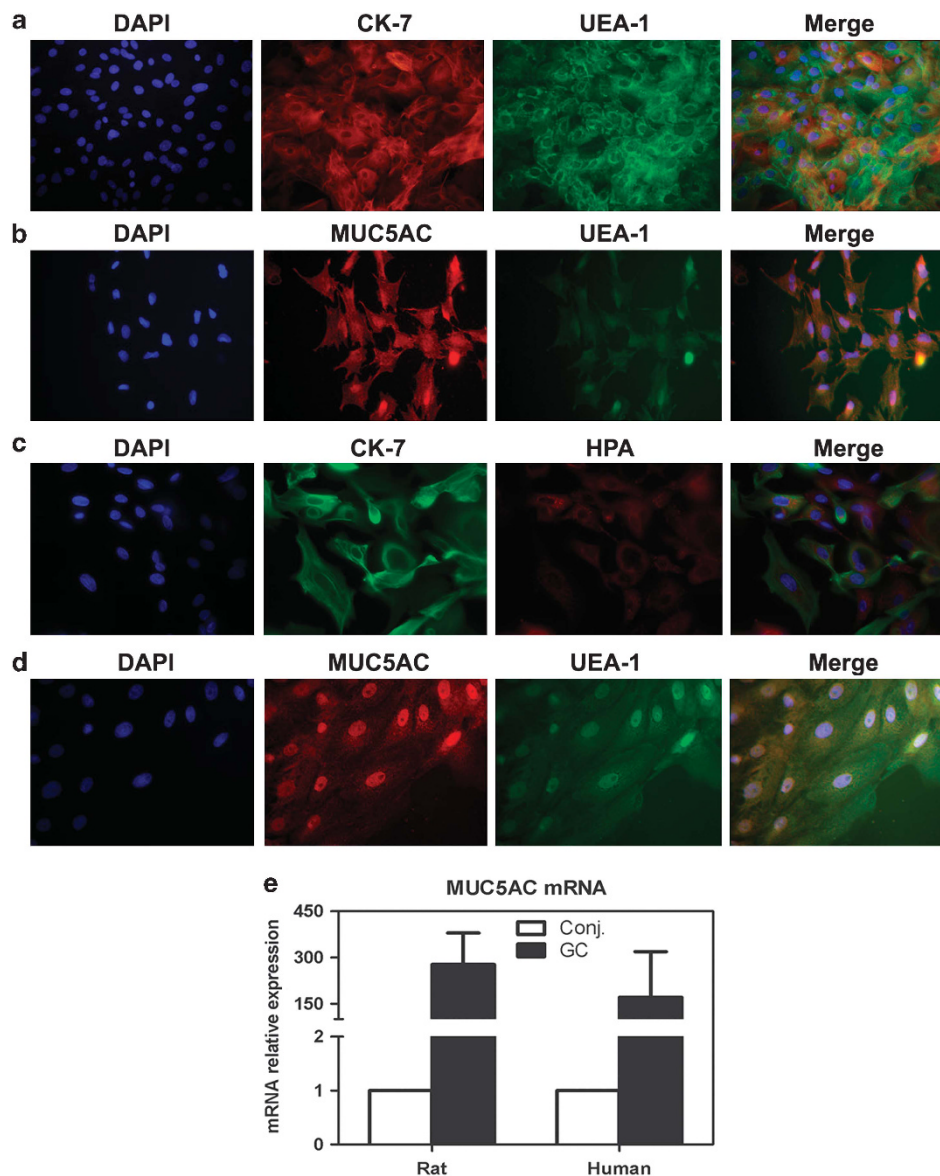

Figure 1 Cultured cells are goblet cells as they contain CK7, lectin binding domains, and MUC5AC. Representative images of rat (**a, b**) and human (**c,d**) cultured goblet cells. Nuclei were stained in blue with DAPI. Cells expressed CK7 (**a,c**), lectins UEA-1 (rat cells, **a**) and HPA (human cells, **c**), and MUC5AC (**b,d**) as seen in merged images. Magnification × 100 for **a,** × 400 for **b** and **d**, and × 200 for **c**. MUC5AC mRNA expression was measured by real-time RT-PCR (**e**). Values are expressed as mean ± s.e.m. (n = 2). CK, cytokeratin; Conj., Conjunctiva; DAPI, 6-diamidino-2-phenylindole; GC, Goblet Cells; HPA, *Helix pomatia agglutinin*; MUC, mucin; RT-PCR, reverse transcription polymerase chain reaction; UEA-1, *Ulex europaeus agglutinin* type 1.

pathway leading to activation of STAT-1 target genes.[16,17] IFN-γ-R can also recruit and activate phosphatidylinositol-3 kinase (PI-3K), Src, or MyD88, that initiate signaling cascades involving ERK1/2, Akt, or NF-κB.[13]

In the conjunctival epithelium, IFN-γ induces squamous metaplasia, which leads to progressive goblet cell loss. Both changes are related to dry eye disease. Recently Zhang et al.[18] showed that IFN-γ caused goblet cell apoptosis in a mouse model of dry eye. However, the signaling pathways activated by this cytokine and its role regulating in goblet cell function remain unclear.

The purpose of this study was to determine whether IFN-γ, a mediator of dry eye, directly regulates mucin production in rat and human conjunctival goblet cells. Thus, the presence of IFN-γ-R-1, as well as the effect on intracellular Ca^{2+} signaling, mucin secretion, and goblet cell proliferation were measured after stimulation with IFN-γ alone or in the presence of a normal stimulus of secretion, a cholinergic agonist. We found that IFN-γ blocked the cholinergic agonist-stimulated increase in $[Ca^{2+}]_i$, mucin secretion, and decreased goblet cell proliferation. Thus, IFN-γ leads to decreased goblet cell mucus production and contributes to the mucin deficiency found in dry eye disease.

RESULTS

Goblet cell characterization

Identity of cells cultured from both rat and human conjunctiva was confirmed by immunofluorescence microscopy using the following markers: cytokeratins (CK) 4 (specific for stratified squamous non-goblet epithelial cells) and 7 (goblet cell specific keratin),[19,20] and lectins from *Ulex europaeus agglutinin* type 1 (UEA-1) (rat) or *Helix pomatia agglutinin* (HPA) (human). Lectins identify high molecular weight glycoconjugates including MUC5AC synthesized and secreted by goblet cells.

The majority of rat cells in culture were positive for both lectin UEA-1 (green) and CK7 (red) as shown in **Figure 1a**. To assure that lectins were identifying goblet cells, immunocytochemistry against UEA-1 and MUC5AC was performed in cultured cells (**Figure 1b**). We observed that UEA-1 and MUC5AC staining co-localized, showing that there were identifying the same mucous products. Human cultured cells were positive for lectin HPA (red) and CK7 (green), as shown in **Figure 1c**, and positive for MUC5AC (**Figure 1d**). Cultured cells did not express CK4 (data not shown). Additionally, the expression of MUC5AC in these cells and in conjunctival tissue from rat and human was confirmed by semi-quantitative real time reverse transcription polymerase chain reaction (RT-PCR) (**Figure 1e**). MUC5AC was detected in both rat (left) and human (right) conjunctival tissue and in cultured goblet cells. MUC5AC mRNA levels were 277.7 times higher in cultured rat goblet cells that in rat conjunctiva, and 170.7 times higher in human goblet cells than in human conjunctiva. Therefore, the overwhelming majority of cells in culture were goblet cells.

Goblet cells express IFN-γ-R1

To determine whether conjunctival goblet cells were able to directly respond to IFN-γ, the presence of IFN-γ-R1, the
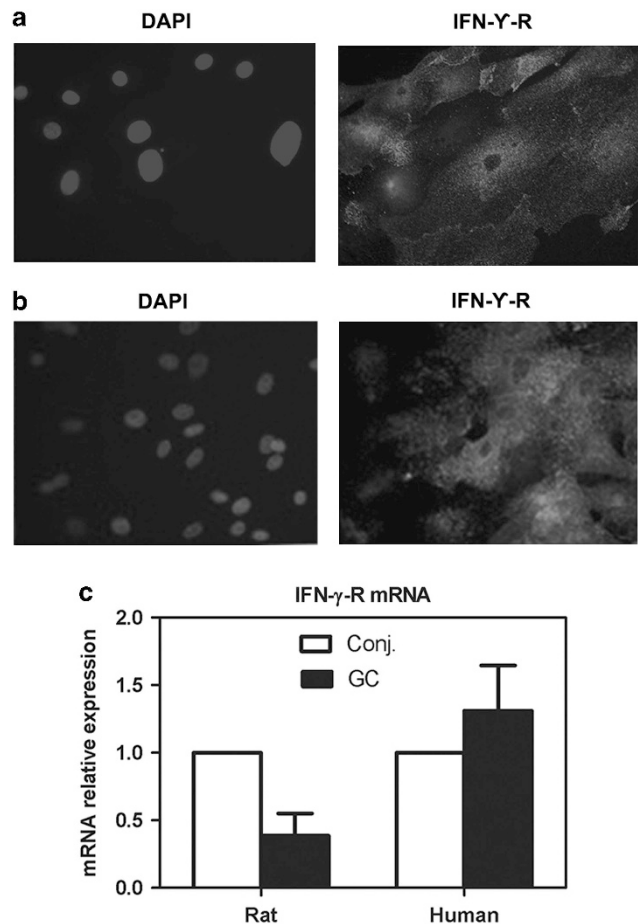

Figure 2 Conjunctival goblet cells express the IFN-γ receptor. Images of rat (**a**) and human (**b**) cultured goblet cells, where IFN-γ-R is stained in red. Nuclei were stained in blue with DAPI. IFN-γ-R mRNA expression was analyzed by real-time RT-PCR (**c**). Values are expressed as mean ± s.e.m. ($n = 2$). Conj., Conjunctiva; DAPI, 6-diamidino-2-phenylindole; GC, Goblet Cells; IFN-γ, interferon-gamma; RT-PCR, reverse transcription polymerase chain reaction.

receptor for this cytokine, was determined by immunofluorescence microscopy and by real-time RT-PCR. IFN-γ-R1 protein expression was identified in both rat and human cultured cells (**Figure 2a,b**). In addition, IFN-γ-R1 was detected in conjunctival tissue and cultured goblet cells (**Figure 2c**). We confirmed that both rat and human cultured cells expressed the receptor needed for IFN-γ to be effective.

IFN-γ increases $[Ca^{2+}]_i$ in goblet cells

Owing to its role in goblet cell signaling, $[Ca^{2+}]_i$ was measured in cultured cells after addition of different stimuli. The effect of IFN-γ was compared with the cholinergic agonist carbachol, an agonist of parasympathetic nerve-mediated responses, that are known to increase $[Ca^{2+}]_i$ in conjunctival goblet cells.[12] Concentration-dependency assays were first performed in cultured rat cells to select optimal concentrations for IFN-γ and carbachol. IFN-γ caused a rapid increase in $[Ca^{2+}]_i$ to a peak value that was maintained at a lower plateau level over time (**Figure 3a**). IFN-γ at 0.1, 1.0, and 3.0 ng ml^{-1} significantly increased the peak $[Ca^{2+}]_i$ in a

concentration-dependent manner to 68.8 ± 29.6, 115.6 ± 37.3, and 239.5 ± 52.3 ($n = 6$), respectively (**Figure 3b**). The highest concentration of IFN-γ $10\,\text{ng}\,\text{ml}^{-1}$ did not produce any change. Thus, IFN-γ itself increases $[Ca^{2+}]_i$. IFN-γ at $3\,\text{ng}\,\text{ml}^{-1}$ was chosen as the maximal concentration for use in subsequent experiments.

When three different concentrations (10^{-6} M, 10^{-5} M, and 10^{-4} M) of carbachol were used, all these concentrations significantly increased $[Ca^{2+}]_i$ over time with a peak response followed by an elevated plateau (**Figure 3c**). When peak values were analyzed, the highest response of 271.4 ± 64.2 nM ($n = 4$) was obtained at 10^{-4} M carbachol (**Figure 3d**). This result is in
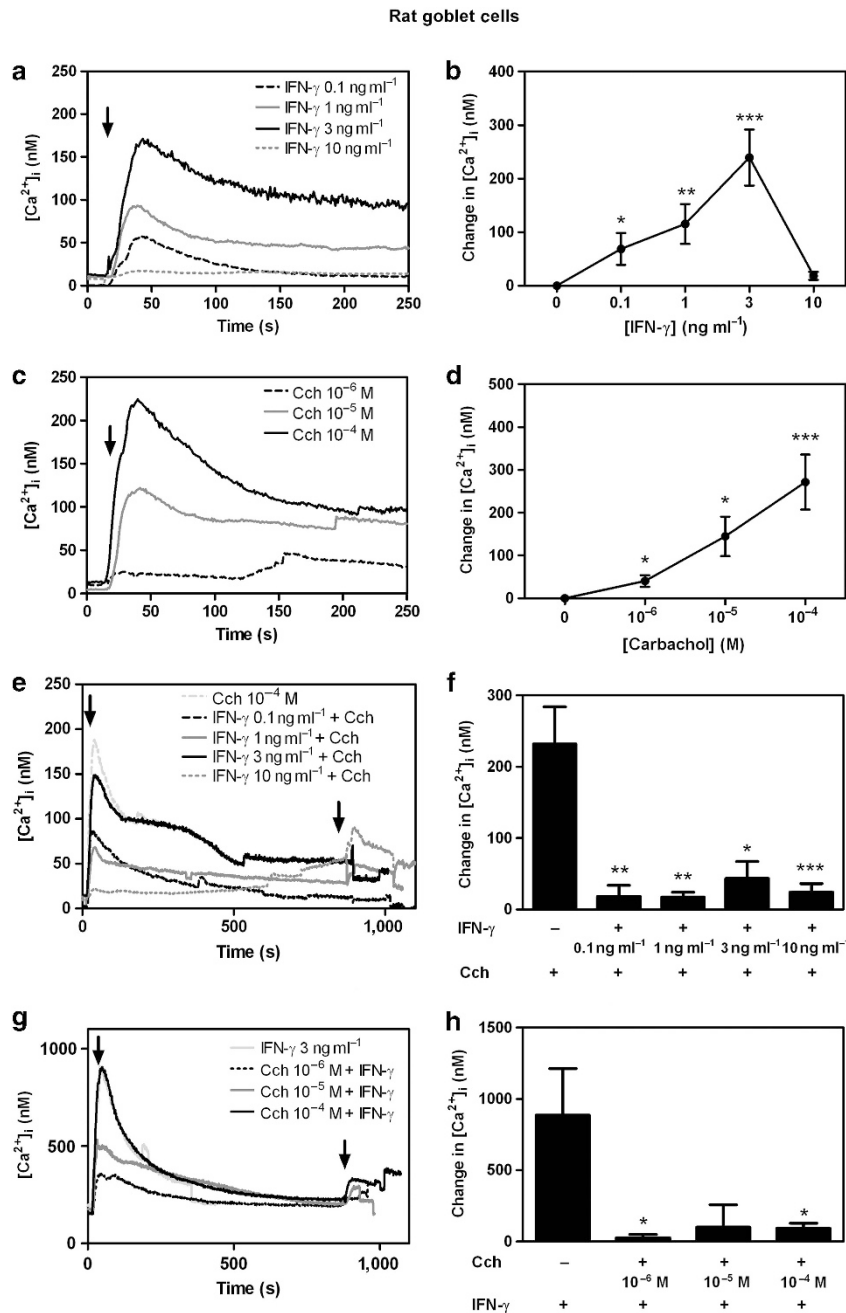

Rat goblet cells

Figure 3 IFN-γ and carbachol cause a concentration-dependent increase in intracellular $[Ca^{2+}]$ and desensitize each other's Ca^{2+} response in cultured conjunctival goblet cells. Concentration dependency of IFN-γ (**a,b**) and Cch (**c,d**) of $[Ca^{2+}]_i$ in rat conjunctival goblet cells. Mean intracellular Ca^{2+} response over time after addition of increasing concentrations of IFN-γ from 0.1 to $10\,\text{ng}\,\text{ml}^{-1}$ ($n = 6$) (**a**) and of Cch from 10^{-6}–10^4 M ($n = 5$) (**c**). Peak $[Ca^{2+}]_i$ over basal is shown in (**b**) and (**d**) for IFN-γ and Cch, respectively. Mean intracellular Ca^{2+} response over time (**e**) or peak value over basal (**f**) in response to Cch at 10^{-4} M alone or the effect of addition of increasing concentrations of IFN-γ (indicated by first arrow) on the Cch response 15 min (indicated by second arrow) after IFN-γ addition ($n = 5$). Mean intracellular Ca^{2+} response over time (**g**) or peak value over basal (**h**) in response to IFN-γ ($3\,\text{ng}\,\text{ml}^{-1}$) alone or the effect of increasing concentrations of Cch (indicated by first arrow) on the IFN-γ response 15 min (indicated by second arrow) after Cch addition ($n = 4$). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$. Cch, carbachol; IFN-γ, interferon-gamma.

agreement with previous studies[21] that found the plateau for carbachol was at 10^{-4} M. Therefore, carbachol at 10^{-4} M was chosen as the concentration to be used in subsequent experiments. Comparison of carbachol and IFN-γ responses shows that IFN-γ caused a Ca^{2+}_i response comparable with that induced by the cholinergic agonist.
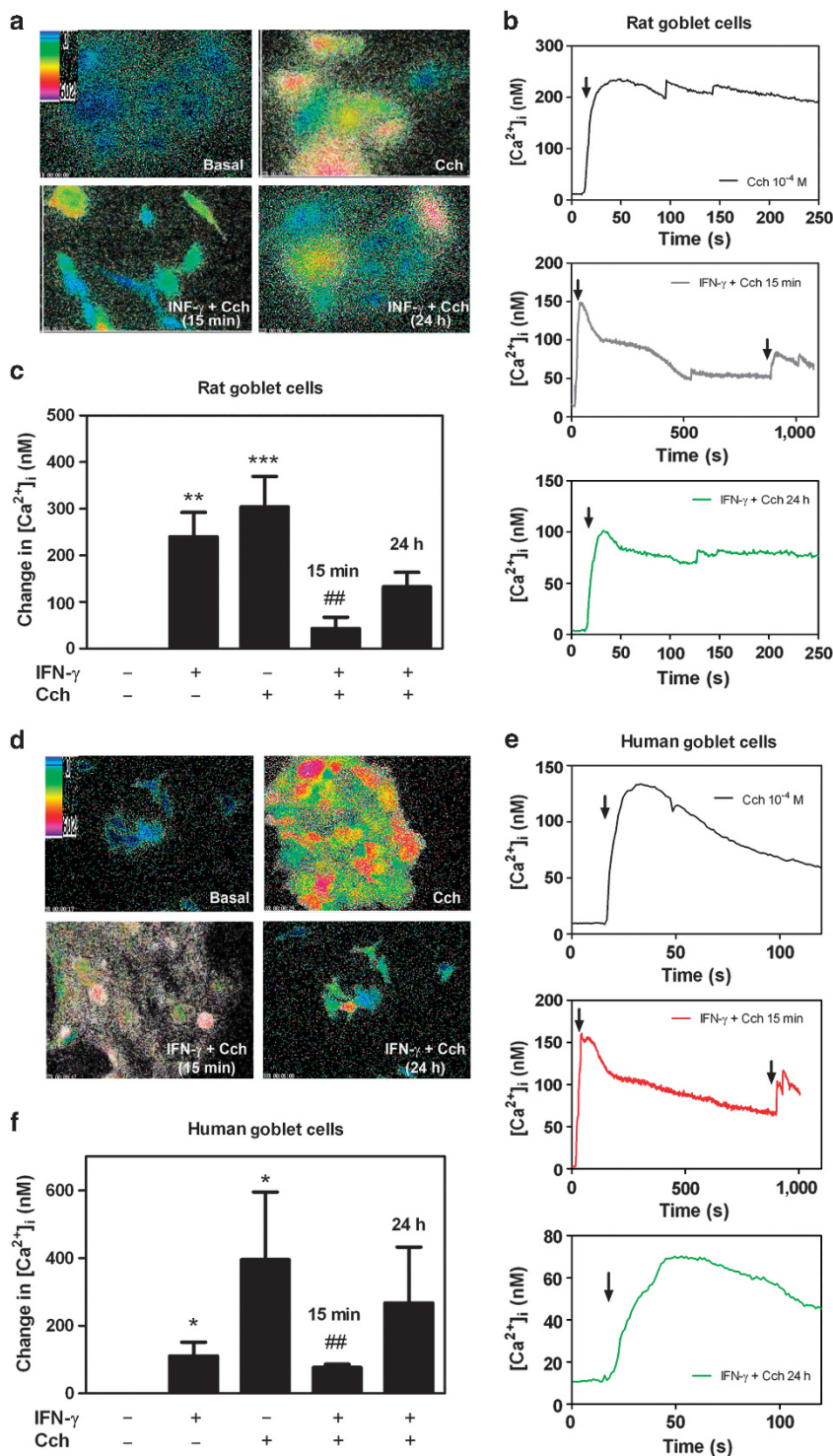

Figure 4 A 15 min, but not a 24 h, incubation with IFN-γ blocks the increase in $[Ca^{2+}]_i$ stimulated by Cch in rat and human conjunctival goblet cells. A pseudo color image of $[Ca^{2+}]_i$ from fura-2 loaded single goblet cells in rat (**a**) and human (**d**) shows the increase in $[Ca^{2+}]_i$ under basal conditions, after stimulation with Cch (10^{-4}M), or Cch after 15 min or 24 h incubation with IFN-γ. Shown in rat (**b**) and human (**e**) goblet cells is the mean intracellular Ca^{2+} response over time, after addition of carbachol (Cch- arrow black line), IFN-γ (first arrow red line), Cch (second arrow red line) after a 15 min treatment with IFN-γ or Cch (first arrow green line) after a 24 h treatment with IFN-γ. Peak $[Ca^{2+}]_i$ over basal for each condition is shown in (**c**) and (**f**) and is expressed as mean ± s.e.m. ($n=5$) in rat cells (**b** and **c**) and ($n=7$) in human cells (**e** and **f**). * indicates statistical significance compared to basal values, and [#] is compared with carbachol values. * or [#]$P \leq 0.05$; ** or [##]$P \leq 0.01$; *** or [###]$P \leq 0.005$. Cch, carbachol; IFN-γ, interferon-gamma.

IFN-γ and a cholinergic agonist block each others' increase in [Ca²⁺]ᵢ in goblet cells

The effect of a 15-min pretreatment with IFN-γ on the carbachol Ca^{2+}_i response was next studied. Carbachol 10^{-4} M significantly increased $[Ca^{2+}]_i$ to 232.0 ± 52.1 nM ($n = 5$) (**Figure 3e,f**). IFN-γ at 0.1, 1.0, 3.0, and 10 ng ml^{-1} significantly inhibited the carbachol-stimulated peak $[Ca^{2+}]_i$ response decreasing it to 18.4 ± 15.6, 17.1 ± 7.1, 43.2 ± 24.0, and 24.2 ± 11.9 nM ($n = 5$), respectively. This was a 92.07%, 92.63%, 81.36%, and 89.57% inhibition, respectively. All four IFN-γ concentrations blocked the carbachol-induced increase in $[Ca^{2+}]_i$ (**Figure 3f**).

Conversely, the effect of 15-min pretreatment with carbachol on the IFN-γ response was also studied. IFN-γ (3 ng ml^{-1}) significantly increased peak $[Ca^{2+}]_i$ to 886.4 ± 326.7 nM ($n = 4$). Carbachol at 10^{-6} M and at 10^{-4} M significantly decreased IFN-γ-stimulated $[Ca^{2+}]_i$ response to 27.3 ± 10.9 and 91.6 ± 19.0 nM ($n = 4$), respectively, with 96.92 and 89.67% inhibition (**Figure 3g,h**). Although not a significant decrease, carbachol at 10^{-5} M decreased the IFN-γ response by 88.6%.

IFN-γ and the cholinergic agonist carbachol each block the intracellular Ca^{2+}_i response of the other agonist.

IFN-γ causes a time-dependent inhibition of a cholinergic agonist-induced increase in [Ca²⁺]ᵢ in goblet cells

The effect of IFN-γ on carbachol-induced increase in $[Ca^{2+}]_i$ was evaluated at two different incubation times. The first time, 15 min, is a short treatment that indicates the immediate effect on intracellular Ca^{2+} levels and the use of the different cellular Ca^{2+} pools. The effect of a 15-min treatment was shown in **Figure 3** and is used as a control in **Figure 4**. The second time, 24 h, is used to study longer-term activation of cytokine-dependent signaling pathways probably involving synthesis of signaling mediators. In rat goblet cells, IFN-γ (3 ng ml^{-1}) increased peak $[Ca^{2+}]_i$ to a mean of 240.4 ± 52.0 nM ($n = 5$) ($P = 0.009$, **Figure 4a–c**). IFN-γ treatment for 15 min significantly reduced the effect of carbachol (10^{-4} M) from 304.5 ± 64.7 nM to 43.2 ± 23.9 nM ($P = 0.009$), whereas with the 24-h treatment, no statistically significant difference was found when comparing the carbachol response before with the response after IFN-γ ($P = 0.06$, **Figure 4a–c**).

In human cells, IFN-γ (3 ng ml^{-1}) also induced a significant increase in peak $[Ca^{2+}]_i$ (**Figure 4d–f**), to an average of 110.7 ± 40.8 nM compared with basal ($n = 7$) ($P = 0.019$; **Figure 4f**). The 15-min treatment with IFN-γ blocked carbachol-induced increase from 395.3 ± 199.8 nM to 76.9 ± 9.0 nM ($P = 0.009$, **Figure 4f**). In contrast, the 24-h treatment with IFN-γ showed no significant effect on the carbachol-induced $[Ca^{2+}]_i$ response before compared with after IFN-γ.

In summary, IFN-γ by itself increased peak $[Ca^{2+}]_i$ in both rat and human cells, although the magnitude of the effect was higher in rat cells. The 15-min, but not the 24-h, treatment with IFN-γ blocked the carbachol-induced increase in $[Ca^{2+}]_i$ in goblet cells from both species. These findings suggest that an alteration in cellular Ca^{2+} handling or activation of similar short-term signaling pathways, but not those longer-term pathways that use transcription factors or protein synthesis, can

regulate the goblet cell intracellular Ca^{2+} response to cholinergic agonists.

Effect of extracellular and intracellular Ca²⁺ store depletion on IFN-γ and cholinergic agonist Ca²⁺ᵢ responses in goblet cells

We explored the cellular Ca^{2+} pools used by IFN-γ and carbachol to increase $[Ca^{2+}]_i$. To determine the role of extracellular Ca^{2+} (Ca^{2+}_o), we removed Ca^{2+}_o for 3 min before adding 3 ng ml^{-1} IFN-γ or 10^{-4} M carbachol. In rat goblet cells, IFN-γ increased $[Ca^{2+}]_i$ to 368.1 ± 109.4 nM ($n = 6$) (**Figure 5a,c**). In the absence of Ca^{2+}_o, this response was significantly decreased to 92.6 ± 41.8 nM. When Ca^{2+}_o was re-added, a significant increase in $[Ca^{2+}]_i$ to 531.9 ± 123.2 nM was observed showing that the intracellular Ca^{2+} stores were not altered in these experiments. Similar results were obtained with carbachol (**Figure 5a,c**). Carbachol increased $[Ca^{2+}]_i$ to 1061.2 ± 356.1 nM ($n = 6$), which was significantly decreased to 258.7 ± 57.7 nM in the absence of Ca^{2+}_o. Re-addition of Ca^{2+}_o increased the carbachol $[Ca^{2+}]_i$ response to 618.9 ± 200.7 nM.

A similar experiment was conducted using human goblet cells (**Figure 5b,d**). In the presence of extracellular Ca^{2+}, IFN-γ increased Ca^{2+} to 398.6 ± 222.7 nM ($n = 5$), and that increase was reduced to 21.2 ± 10.6 nM in the absence of Ca^{2+}_o. When Ca^{2+}_o was re-added, $[Ca^{2+}]_i$ increased to 380.5 ± 225.0. Carbachol increased $[Ca^{2+}]_i$ to 394.4 ± 77.9 nM, and in the absence of Ca^{2+}_o, it was decreased to 100.7 ± 34.8 nM. Re-addition of Ca^{2+}_o increased $[Ca^{2+}]_i$ to 284.2 ± 215.4 nM.

To explore the role of intracellular Ca^{2+} stores, rat goblet cells were treated for 15 min with 10^{-5} M thapsigargin or for 30 min with 10^{-5} M 2-amino-ethoxydiphenylborane (2-APB). Thapsigargin depletes intracellular Ca^{2+} stores and 2-APB blocks store-operated calcium release.[22] After treatment, cells were stimulated with 3 ng ml^{-1} IFN-γ or 10^{-4} M carbachol (**Figure 5e–h**). IFN-γ increased $[Ca^{2+}]_i$ to 961.4 ± 308.1 nM ($n = 6$) (**Figure 5e,g**). The IFN-γ-induced increase in $[Ca^{2+}]_i$ after treatment with thapsigargin was significantly reduced to 110.5 ± 66.6 and with 2-APB was decreased to 303.3 ± 146.7 nM. Carbachol increased $[Ca^{2+}]_i$ to 1128.9 ± 348.6 nM ($n = 6$) (**Figure 5f,h**). After treatment with thapsigargin, the carbachol increase in $[Ca^{2+}]_i$ was significantly depressed to 212.1 ± 131.2 and after 2-APB was decreased to 422.5 ± 217.5 nM.

Both IFN-γ and carbachol responses were significantly lowered with prior treatment with thapsigargin. 2-APB also blocked both IFN-γ and carbachol responses, but the decrease was not statistically significant. IFN-γ and cholinergic agonists use similar cellular Ca^{2+} stores in goblet cells. These stores have both intracellular and extracellular components as previously demonstrated for cholinergic agonists.[23]

Effect of inhibition of PI-3K and ERK1/2 on IFN-γ- and cholinergic agonist-stimulated increase in [Ca²⁺]ᵢ in goblet cells

Phosphoinositide-3 kinase (PI-3K) and mitogen-activated protein kinase kinase (MEK) 1/2 form part of an alternative IFN-γ signaling cascade compared with STAT-1.[13] Rat goblet cells were preincubated with the PI-3K inhibitor LY294002 or
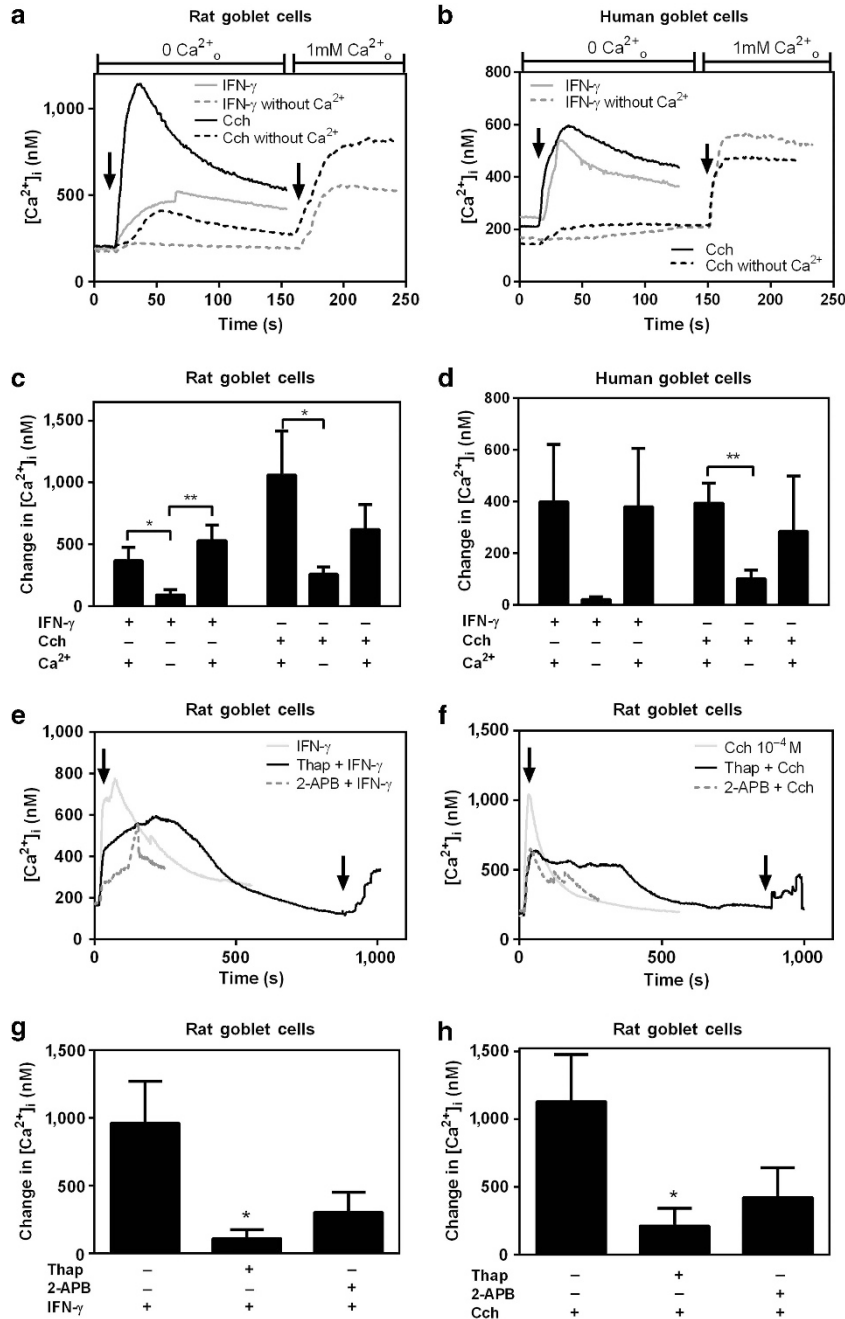
Figure 5 The IFN-γ and Cch stimulated increases in $[Ca^{2+}]_i$ are dependent upon both extracellular Ca^{2+} and intracellular Ca^{2+} stores in rat and human conjunctival goblet cells. The mean $[Ca^{2+}]_i$ over time is shown for 3 ng ml^{-1} IFN-γ or 10^{-4} M Cch in the presence or absence of extracellular calcium (first arrow), or the re-addition of extracellular Ca^{2+} (second arrow) was studied in rat (**a**) and human (**b**) goblet cells in ($n = 6$ in rat cells) or ($n = 5$ in human cells). Peak $[Ca^{2+}]_i$ over basal for each condition is shown in (**c**) and (**d**) and is expressed as mean $\pm$ s.e.m. The $[Ca^{2+}]_i$ over time is shown for 3 ng ml^{-1} IFN-γ (**e**) or 10^{-4} M Cch (**f**) alone (first arrow) or after a 15-min treatment with thapsigargin (10^{-5} M) (second arrow) or 30 min after addition of 2-APB (10^{-5} M) in rat goblet cells ($n = 6$). Peak $[Ca^{2+}]_i$ over basal for each condition is shown in (**g**) and (**h**) and is expressed as mean $\pm$ s.e.m. *$P \leq 0.05$; **$P \leq 0.01$. 2-APB, 2-amino-ethoxydiphenylborane; Cch, carbachol; IFN-γ, interferon-gamma.

the MEK1/2 inhibitor U0126 at 10^{-5} M (prevents activation of ERK1/2) for 30 min and then stimulated with IFN-γ (3 ng ml^{-1}) or carbachol (10^{-4} M).

IFN-γ increased $[Ca^{2+}]_i$ to 453.7 $\pm$ 82.9 nM ($n = 5$) (**Figure 6a,b**). Addition of LY294002 before IFN-γ decreased $[Ca^{2+}]_i$ to 259.3 $\pm$ 130.3 nM, but the effect was not significant.

When U0126 was added, goblet cell response to IFN-γ was significantly reduced to 133.9 $\pm$ 68.5 nM. This is a 70.49% inhibition.

Carbachol increased $[Ca^{2+}]_i$ to 942.9 $\pm$ 253.4 nM ($n = 5$) (**Figure 6c,d**). After pretreatment with LY294002, the response to carbachol was significantly decreased to 114.3 $\pm$ 10.7 nM, an
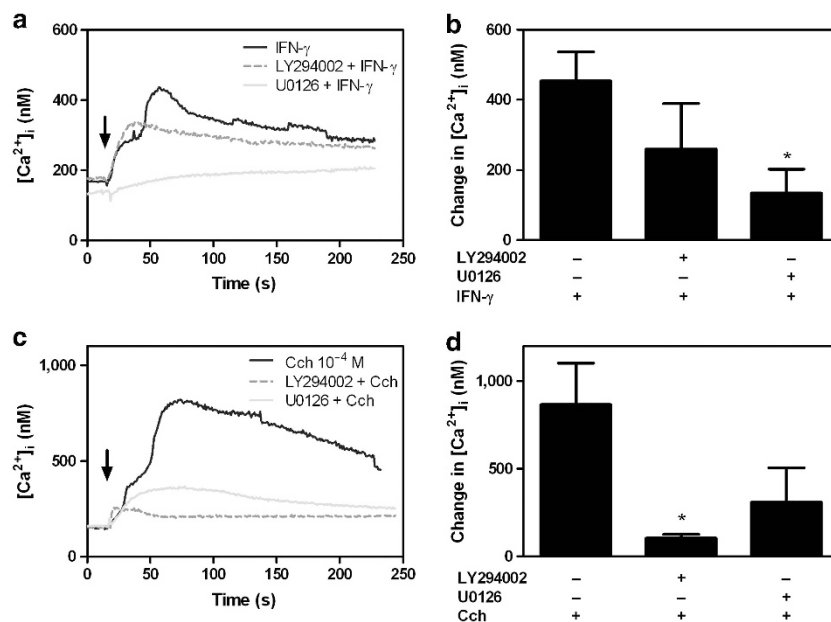
Figure 6 Blockage of PI-3K or ERK1/2 differentially inhibits IFN-γ and Cch stimulation of [Ca^{2+}]$_i$. The mean [Ca^{2+}]$_i$ over time is shown for 3 ng ml^{-1} IFN-γ (**a**) or 10^{-4} M Cch (**c**) alone or after a 30 min treatment with the PI-3K inhibitor LY294002 (10^{-5} M) or the MEK inhibitor U0126 (10^{-5} M) in rat goblet cells ($n = 3$). Arrow indicates addition of agonist. Peak [Ca^{2+}]$_i$ over basal for each condition is shown in (**b**) and (**d**) and is expressed as mean ± s.e.m. *$P \leq 0.05$. Cch, carbachol; ERK, extracellular regulated kinase; IFN-γ, interferon-gamma; MEK, mitogen-activated protein kinase kinase; PI-3K, phosphatidylinositol-3 kinase.

87.88% inhibition. In contrast, preincubation with U0126 did not significantly inhibit carbachol-mediated increase in [Ca^{2+}]$_i$, which was 309.0 ± 197.4 nM.

These data suggest that in goblet cells, IFN-γ induces ERK1/2, but not PI-3K to increase [Ca^{2+}]$_i$. In contrast, cholinergic agonists activate PI-3K, but not ERK1/2 to increase [Ca^{2+}]$_i$. These results confirm results from a previous study.[24]

IFN-γ induces mucin secretion and blocks cholinergic agonist-stimulated mucin secretion from goblet cells

Goblet cell secretion was evaluated after stimulation with 3 ng ml^{-1} IFN-γ or 10^{-4} M carbachol. IFN-γ by itself did not have a significant effect on rat goblet cell secretion, and did not stimulate secretion from human cells ($n = 3$) (**Figure 7a,d**). As a positive control, carbachol induced high molecular weight glycoconjugate secretion from both rat and human goblet cells to 1.60 ± 0.08 and 2.01 ± 0.20-fold increase over basal ($n = 3$), respectively. This response is consistent with previous results.[25] The effect of IFN-γ on carbachol-induced secretion was determined at two times of incubation 15 min and 24 h. A 15-min treatment with IFN-γ did not have any significant effect on carbachol-induced secretion, in either rat or human goblet cells. In contrast, IFN-γ incubated for 24 h significantly blocked carbachol-induced secretion to 0.88 ± 0.06 and to 1.21 ± 0.23 in rat ($P = 0.002$, **Figure 7b**) and human cells ($P = 0.05$, **Figure 7c**), respectively. In rat cells, a 120.0% inhibition was obtained, whereas in human cells, a 79.2% inhibition was obtained.

These data suggest that activation of a long-term pathway such as JAK-STAT by IFN-γ can prevent cholinergic agonist-stimulated secretion, but that stimulating a short-term pathway such as increasing the [Ca^{2+}]$_i$ cannot.

IFN-γ decreases goblet cell proliferation

Cell proliferation was measured in rat conjunctival goblet cells. A 24-h treatment with IFN-γ at 3 ng ml^{-1} decreased proliferation to 0.8 compared with basal value set as 1 ($n = 3$) ($P = 0.00018$) (**Figure 8**). As expected, carbachol 10^{-4} M did not have a significant effect on goblet cell proliferation after a 2 h treatment and IFN-γ added 24 h before carbachol did not alter proliferation either.

DISCUSSION

The role of the Th1 cytokine IFN-γ has been widely studied in a number of diseases, such as dry eye, Steven-Johnson syndrome, or Sjögren's syndrome, where elevated levels of IFN-γ have been found.[26–28] In humans, IFN-γ correlates with disease severity.[29] Our results demonstrate that IFN-γ, which is found to be increased in several inflammatory diseases of the conjunctiva, has a direct effect on conjunctival goblet cell function. We found that IFN-γ increases [Ca^{2+}]$_i$, but does not stimulate goblet cell secretion. As a decrease in goblet cell mucin production has a critical role in ocular surface inflammation,[30] the most important finding of the present study is that IFN-γ blocks two of the three processes used by goblet cells to increase mucin production: mucin secretion and goblet cell proliferation. That is, IFN-γ prevented the increase in [Ca^{2+}]$_i$ and stimulation of secretion caused by cholinergic agonists. In addition, IFN-γ itself reduced goblet cell proliferation. These *in vitro* studies using isolated goblet cells were performed to determine the effect of IFN-γ on goblet cells not contaminated

a

Rat goblet cells

(Bar chart: Amount of glycoconjugate secretion (fold increase above basal) vs IFN-γ / Cch conditions, 15 min)

b

Rat goblet cells

(Bar chart with * and ### annotations, 24 h)

c

Human goblet cells

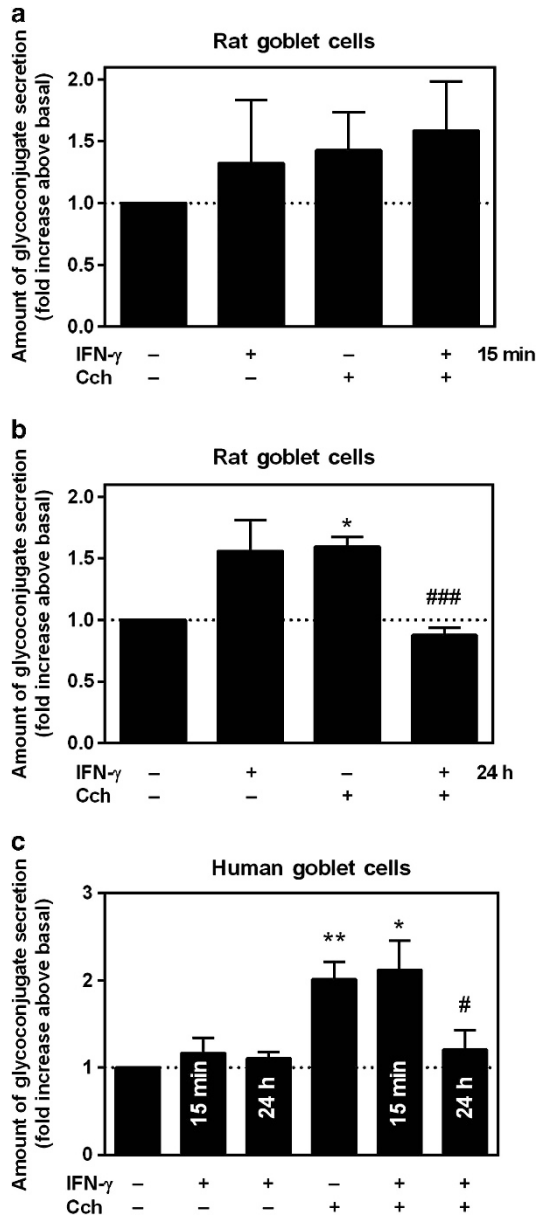(Bar chart with **, *, # annotations; bars labeled 15 min and 24 h)

Figure 7 IFN-γ blocks Cch stimulated secretion of high molecular weight glycoproteins in rat and human goblet cells. Secretion from rat (**a,b**) and human (**c**) goblet cells is shown after a 2-h incubation with Cch (10^{-4} M), a 24-h incubation with IFN-γ (3 ng ml^{-1}), or a 15 min or 24 h treatment with IFN-γ followed by a 2-h incubation with Cch. Data are mean ± s.e.m. ($n = 3$). * means statistical significance compared with basal values, and # is compared with Cch values. * or #$P \leq 0.05$; ** or ##$P \leq 0.01$; *** or ###$P \leq 0.005$. Cch, carbachol; IFN-γ, interferon-gamma.

by other cell types. *In vivo*, is likely that cross talk between goblet and non-goblet cells occurs, involving multiple signaling cascades that could alter goblet cell signaling pathways. Therefore, further research *in vivo* is warranted.

Cultured goblet cells responded to IFN-γ in a concentration-dependent manner. IFN-γ from 0.1 to 3 ng ml^{-1} showed progressive increase in $[Ca^{2+}]_i$, whereas the highest concentration (10 ng ml^{-1}) did not. This suggests that at the highest concentration of IFN-γ, a Ca^{2+} inhibitory signaling pathway is

Goblet cell proliferation

(Bar chart: Goblet cell proliferation (fold increase over basal) vs IFN-γ / Cch conditions, with *** annotation)
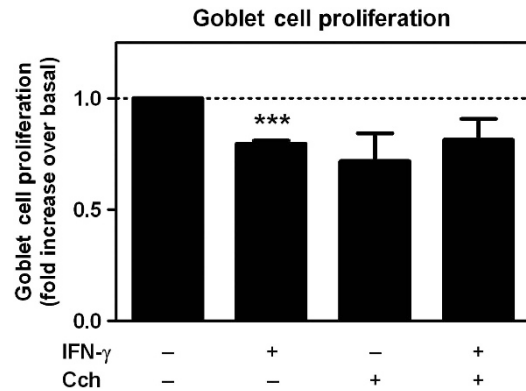
Figure 8 IFN-γ inhibits proliferation of cultured rat conjunctival goblet cells. Proliferation of goblet cells in response to no addition, IFN-γ (3 ng ml^{-1}) for 24 h, Cch (10^{-4} M) for 2 h, alone or after IFN-γ for 24 h. Data are mean ± s.e.m. ($n = 3$). ***$P \leq 0.005$. Cch, carbachol; IFN-γ, interferon-gamma.

being activated. Interestingly, all four concentrations of IFN-γ blocked the effect of cholinergic agonists in a very similar way. This result suggests that IFN-γ at high concentrations is not using a Ca^{2+}-dependent mechanism to block cholinergic agonist-mediated Ca^{2+} responses. IFN-γ is known to activate other short-term signaling pathways[16] that will be investigated in future.

The mechanisms involved in short- and long-term responses induced by IFN-γ are likely to be different. Short-term blockade could be due to depletion of intracellular calcium reservoirs. Our results suggest that cholinergic agonists and IFN-γ are using, at least in part, the same intracellular Ca^{2+} reservoirs. Major Ca^{2+} reservoirs are the intracellular stores located in the endoplasmic reticulum[31] that are linked by Orai-1 and STIM1 to Ca^{2+} influx.[32,33] This intracellular store is depleted by thapsigargin. The findings that: (i) intracellular Ca^{2+} responses caused by cholinergic agonists and IFN-γ are both decreased after the addition of thapsigargin, and (ii) the recovery of the intracellular Ca^{2+} response after the re-addition of extracellular Ca^{2+} support this hypothesis. In agreement with our hypothesis is the finding that cholinergic agonist treatment for 15 min also inhibited IFN-γ-induced Ca^{2+} responses and that a similar IFN-γ treatment inhibited the cholinergic Ca^{2+} response.

Cholinergic agonists bind to muscarinic receptors and IFN-γ binds to its own receptor, IFN-γ-R. The main signaling pathway activated by IFN-γ-R is JAK-STAT,[17] a pathway probably not activated by muscarinic receptors. However, several of the alternative pathways activated by IFN-γ involve two kinases that form part of the muscarinic pathways, PI-3K and MEK1/2,[16] suggesting that an interaction based on these pathways may be occurring. For that reason, we used specific inhibitors of these two kinases. We found that when PI-3K was blocked, the response to cholinergic agonists was inhibited, but the response to IFN-γ was not altered. In contrast, when MEK1/2 was inhibited, goblet cell response to cholinergic agonists remained unaltered, whereas response to IFN-γ was significantly blocked. Even though cholinergic agonists and IFN-γ use

Table 1 Primer sequences

Primer	Primer sequence/Reference	Source
GAPDH	Sense: 5′-GAACGTGAAGGTCGGAGTCAAC-3′	Sigma-Aldrich (St Louis, MO)
	Antisense: 5′-CGTGAAGATGGTGATGGGATTTC-3′	
MUC5AC	Sense: 5′-CCCACAGAACCCAGTACAA -3′	Sigma-Aldrich
	Antisense: 5′-AATGTGTAGCCCTCGTCT -3′	
Human IFN-γ-R1	Ref: HP200396	OriGene Technologies, Inc. (Rockville, MD)
Rat IFN-γ-R1	Ref. PPR06409A	Qiagen (Frederick, MD)

the same cellular Ca^{2+} stores, the mechanism by which they use these stores appears to be different. The short-term effect could also be independent of Ca^{2+}, occur before the rise in intracellular Ca^{2+}, or involve a direct effect on the muscarinic receptors. Because both IFN-γ and cholinergic agonists blocked one another's responses, a cross desensitization of both receptors may be occurring.

A hallmark of dry eye disease is reduced mucin secretion. Although the levels of $[Ca^{2+}]_i$ were not significantly blocked after 24-h incubation with IFN-γ alone, secretion induced by cholinergic agonists was reduced when there was a previous 24-h incubation with IFN-γ. This result is similar to the report of Contreras-Ruiz et al.[34] of a reduction of cholinergic agonist-mediated mucin secretion of mouse goblet cells exposed to IFN-γ for 24 h. As a 24-h treatment of IFN-γ does not alter cholinergic agonist increase in $[Ca^{2+}]_i$, it is possible that activation of the JAK-STAT pathway that involves stimulation of transcription factors and synthesis of proteins could be responsible for the inhibition of secretion.

We showed in this study that IFN-γ decreases cultured goblet cell proliferation. This conclusion is supported by published results that associated increased IFN-γ levels with low numbers of goblet cells and increased apoptosis.[18,35] Interestingly, in some cells, IFN-γ stimulation led to JAK2-dependent trans-activation of epidermal growth factor receptor.[13] As activation of the epidermal growth factor receptor is known to stimulate conjunctival goblet cell proliferation, it is unlikely that the JAK-STAT pathway has a role in the IFN-γ blockade of cell proliferation. Blockade of goblet cell proliferation induced by IFN-γ could use another long-term mechanism or signaling pathway different from that used to decrease secretion.

A decrease in goblet cell proliferation could result from inhibition of the cell cycle or stimulation of apoptosis. When IFN-γ is present in the goblet cell environment, the proliferation of these cells is blocked (current study) and the apoptotic processes begin, as demonstrated by Zhang et al.[18] IFN-γ has been involved in goblet cell apoptosis through both extrinsic and intrinsic apoptosis pathways.[18] The main organelles that regulate Ca^{2+} homeostasis are also the main sites of apoptotic regulation.[36] Thus, there could be an interaction between apoptotic and other Ca^{2+} pathways that explain the long-term effect on IFN-γ on proliferation.

In previous studies, rat conjunctival goblet cells were found to behave in a similar manner as human goblet cells.[37–39]

Similarly, in the current study in both rat and human goblet cells, IFN-γ blocked carbachol-mediated increase in $[Ca^{2+}]_i$ and secretion in both species. Thus, rat conjunctival goblet cells are an excellent model for human goblet cells, especially considering the difficulty in obtaining human conjunctiva.

The main limitation of using primary cultures is the high variability between experiments. We observed that especially in calcium experiments, where only a few cells are analyzed, there is an increased risk of higher variation. In addition, in the case of human cells, that variability may be increased because of the use of cells from both male and females, as well as from elderly donors. However, even with those limitations, we found several significant effects.

We conclude that IFN-γ affects multiple processes that control the amount of mucin produced by goblet cells. IFN-γ itself decreases goblet cell number by blocking proliferation and potentially by stimulating apoptosis. In addition, we have shown that the normal goblet cell increase in $[Ca^{2+}]_i$ and mucin secretion, usually mediated by cholinergic agonists, is blocked by this Th1 cytokine. Taking all these data together, we conclude that the presence of IFN-γ could explain the mucin deficiency typically found in dry eye disease[6] because goblet cell number is diminished and the secretion of the remaining cells is blocked.

METHODS

Materials. RPMI-1640 cell culture medium, penicillin/streptomycin, and L-glutamine were purchased from Lonza (Walkerville, IL). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA).

Antibodies against CK4, CK7, and MUC5AC were from Abcam (Cambridge, MA). The antibody against IFN γ receptor was from Novus Biologicals (Littleton, CO). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Lectins UEA-1 and HPA were from Sigma-Aldrich (St Louis, MO).

Primers for human IFN-γ receptor were from OriGene Technologies (Rockville, MD), and those for rat were from SABioscience-Qiagen (Frederick, MD). The Superscript First-Strand Synthesis system for RT-PCR was from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix was from Applied Biosystems (Carlsbad, CA).

Recombinant Rat IFN-γ and recombinant Human IFN-γ (carrier-free) were purchased from BioLegend (San Diego, CA). Carbachol was purchased from Sigma (St Louis, MO). Fura-2/AM was from Life Technologies (Grand Island, NY). The 35 mm Glass Bottom Culture Dishes were from MatTek Corporation (Ashland, MA). Thapsigargin and 2-APB were from Sigma-Aldrich, and LY294002 and U0126 were from Tocris Bioscience (Minneapolis, MN).

Enzyme-linked lectin assay was from Pierce Biotechnology (Rockford, IL) and Amplex Red was from Invitrogen. The Cell

Counting Kit-8 was purchased from Dojindo Molecular Technologies (Gaithersburg, MD).

Animals. Male Sprague-Dawley rats between 4 and 5 weeks of age were obtained from Taconic Farms (Germantown, NY). All experiments followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Rats were anesthetized with CO_2 for 2 min and then killed by decapitation. Forniceal and bulbar conjunctival tissues were removed from both eyes.

Human tissue. Human conjunctival tissues were obtained from Heartland Lions Eye Bank (Columbia, MO) and Michigan Eye Bank (Ann Arbor, MI). This study adhered to the Tenets of the Declaration of Helsinki and was approved by the Schepens Eye Research Institute Human Studies Internal Review Board. Received tissues were normal bulbar and forniceal conjunctival tissues.

Cell culture. Tissue samples were obtained from 29 rats and from 12 human donors. Rat and human conjunctival goblet cells were grown in organ culture as previously described.[37,40] Briefly, conjunctival tissue was carefully minced into small pieces, and placed in six-well plates. When cell outgrowth was observed, tissue explants were removed. Conjunctival cells were cultured from every sample, although goblet cells were not obtained from each piece. As early as 24 h after establishment of organ culture, cell outgrowth from the explant was observed. Cells were fed with RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 µg ml^{-1} penicillin-streptomycin. Cells were maintained at 37 °C in 5% CO_2, and the medium was changed every other day. After 7–10 days, cells were trypsinized and passaged. Cells in passage 1 were used for all experiments.

Immunofluorescence microscopy. First-passage cultured cells were grown on glass cover slips and fixed in methanol or formaldehyde. To confirm that the cultured cells were goblet cells, these cells were stained with antibody against CK7, CK4, MUC5AC, and the lectins UEA-1 conjugated to FITC or HPA conjugated to tetramethylrhodamine. Cells were incubated for 2 h with a blocking solution. Thereafter, primary antibody anti-CK7, anti-CK4, or anti-MUC5AC at 1:100 dilution was added. After 1 h, cover slips were washed with phosphate-buffered saline, and then secondary antibody conjugated with Cy2 or Cy3 (at 1:200 dilution), and UEA-1 or HPA (at 1:500 dilution) were added for 1 h. To detect cell nuclei, 6-diamidino-2-phenylindole was added to the mounting medium. The same protocol was used to determine the presence of IFN-γ receptor, using an anti-IFN-γ-R antibody. Negative controls included the omission of primary antibodies. Specificity of primary antibodies and lectins had been previously tested. Cells were viewed by fluorescence microscopy (Eclipse E80i, Nikon, Tokyo, Japan). Micrographs were taken with a digital camera (Spot, Diagnostic Instruments, Sterling Heights, MI).

RNA isolation and real-time reverse transcript PCR (RT^2-PCR). Briefly, RNA was extracted with TRIzol and total RNA was isolated according to the manufacturer's instructions. One microgram of purified total RNA was used for complementary DNA synthesis using the Superscript First-Strand Synthesis system for RT-PCR.

RT^2-PCR reaction was performed with 10 ng complementary DNA, 1 µl primers (**Table 1**), and 10 µl SYBR Green PCR Master Mix in a final volume of 20 µl. Conditions of the PCR reaction were: denaturation at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. After the 40 cycles, there was a final cycle of 95 °C for 90 s. All reactions were performed in duplicate. The levels of GAPDH for each sample were used as endogenous controls. Non-template controls included the omission of complementary DNA. To assure the specificity of the PCR products, a melting curve analysis was performed. For the posterior analysis of mRNA expression levels, the $2^{-\Delta\Delta Ct}$ method was used.[41]

Measurement of $[Ca^{2+}]_i$. First-passage cultured goblet cells were grown on 35-mm glass-bottom culture dishes for 1 day. Cells were then incubated in KRB buffer (containing 120 mM NaCl, 25 mM NaHCO$_3$, 10 mM HEPES, 4.8 mM KCl, 1.2 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$, 1 mM CaCl$_2$) with 0.5% bovine serum albumin, 8 µM pluronic acid F127, 250 µM sulfinpyrazone, and 0.5 µM of Fura-2/AM for 1 h at 37 °C. Fura-2/AM is a fluorescent molecule that indicates the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). After incubation, cells were washed with KRB buffer containing sulfinpyrazone, and the dishes were observed using a Ca^{2+} imaging system, InCyt Im2 (Intracellular Imaging, Cincinnati, OH). This system allows measuring the ratio of Fura-2 using excitation wavelengths of 340 and 380 nm, and an emission wavelength of 505 nm. A mean of 10 cells per dish was selected, and $[Ca^{2+}]_i$ was measured in each individual cell. A basal reading was done for at least 15 s before addition of agonists or inhibitors. Data are presented as the change in peak $[Ca^{2+}]_i$, that was calculated by subtracting the basal value from the $[Ca^{2+}]_i$ peak.

High molecular weight glycoconjugate secretion. For secretion assays, first-passage goblet cells were cultured in 24-well plates and grown to confluence. After serum starving for 24 h, cells were incubated with buffer alone (basal), carbachol (10^{-4} M), or IFN-γ (3 ng ml^{-1}) for 24 h in serum-free RPMI 1640 supplemented with 0.5% bovine serum albumin. Goblet cell secretion was measured using enzyme-linked lectin assay. UEA-1 lectin conjugated to horseradish peroxidase was used to detect high molecular weight glycoconjugates, including the mucin MUC5AC produced by rat and human goblet cells. After incubation, the culture medium was collected and the amount of lectin-detectable glycoconjugates was measured. After collection of supernatant for the enzyme-linked lectin assay, cells in the 24-well plate were removed and sonicated. The cell homogenate was analyzed for total amount of protein using the Bradford protein assay. Bovine submaxillary mucin was used for the standard curve.

To perform the enzyme-linked lectin assay, standards and supernatants were placed into 96-well microplates and dried overnight at 60 °C. The manufacturer's protocol was followed. UEA-1 was detected using Amplex Red. In the presence of hydrogen peroxide, Amplex Red is oxidized producing a fluorescent molecule. Fluorescence was then quantified using a fluorescence ELISA reader (Bio-Tek, Winooski, VT), using 530 and 590 nm excitation and emission wavelengths, respectively. The amount of high molecular weight glycoconjugate secretion was normalized to total protein in the homogenate, and expressed as fold increase over basal. Basal value was set at 1.

Proliferation. Proliferation was measured using Cell Counting Kit-8. Briefly, cells were serum starved for 24 h, treated with IFN-γ (3 ng ml^{-1}), for 24 h, and then stimulated with carbachol (10^{-4} M) for 2 h. After stimulation, WST-8 product was added to the wells and after a 45-min incubation, absorbance was read in a spectrophotometer, following manufacturer's instructions.

Data presentation and statistical analysis. Data were presented as mean ± standard error of the mean (s.e.m.). Student's t-test was performed to analyze data and $P \leq 0.05$ was considered statistically significant.

DISCLOSURE
The authors declared no conflict of interest.

REFERENCES

1. Turner, J. & Jones, C.E. Regulation of mucin expression in respiratory diseases. *Biochem. Soc. Trans.* **37**, 877–881 (2009).
2. Kim, Y.S. & Ho, S.B. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr. Gastroenterol. Rep* **12**, 319–330 (2010).
3. Dartt, D.A. Control of mucin production by ocular surface epithelial cells. *Exp. Eye Res.* **78**, 173–185 (2004).
4. Floyd, A.M. *et al.* Mucin deficiency causes functional and structural changes of the ocular surface. *PLoS One* **7**, e50704 (2012).
5. Argueso, P. & Gipson, I.K. Epithelial mucins of the ocular surface: structure, biosynthesis and function. *Exp. Eye Res.* **73**, 281–289 (2001).
6. Argueso, P., Balaram, M., Spurr-Michaud, S., Keutmann, H.T., Dana, M.R. & Gipson, I.K. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. *Invest. Ophthalmol. Vis. Sci.* **43**, 1004–1011 (2002).
7. Barabino, S. & Rolando, M. Amniotic membrane transplantation elicits goblet cell repopulation after conjunctival reconstruction in a case of severe ocular cicatricial pemphigoid. *Acta Ophthalmol. Scand.* **81**, 68–71 (2003).
8. Kunert, K.S., Tisdale, A.S. & Gipson, I.K. Goblet cell numbers and epithelial proliferation in the conjunctiva of patients with dry eye syndrome treated with cyclosporine. *Arch. Ophthalmol.* **120**, 330–337 (2002).
9. Lemp, M.A. Basic principles and classifications of dry eye disorders. In *The Dry Eye: A Comprehensive Guide* (Lemp, M.A. & Marquandt, R., eds) 101–131 (Springer, New York, 1992).
10. Hodges, R.R., Horikawa, Y., Rios, J.D., Shatos, M.A. & Dartt, D.A. Effect of protein kinase C and Ca(2 +) on p42/p44 MAPK, Pyk2, and Src activation in rat conjunctival goblet cells. *Exp. Eye Res.* **85**, 836–844 (2007).
11. Decrock, E. *et al.* IP3, a small molecule with a powerful message. *Biochim. Biophys. Acta* **1833**, 1772–1786 (2013).
12. Dartt, D.A. *et al.* Regulation of conjunctival goblet cell secretion by Ca(2 +)and protein kinase C. *Exp. Eye Res.* **71**, 619–628 (2000).
13. Adler, K.B., Tuvim, M.J. & Dickey, B.F. Regulated mucin secretion from airway epithelial cells. *Front. Endocrinol. (Lausanne)* **4**, 129 (2013).
14. Boehm, U., Klamp, T., Groot, M. & Howard, J.C. Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* **15**, 749–795 (1997).
15. Farrar, M.A. & Schreiber, R.D. The molecular cell biology of interferon-gamma and its receptor. *Annu. Rev. Immunol.* **11**, 571–611 (1993).
16. Gough, D.J., Levy, D.E., Johnstone, R.W. & Clarke, C.J. IFNgamma signaling-does it mean JAK-STAT? *Cytokine Growth Factor Rev.* **19**, 383–394 (2008).
17. Hu, X. & Ivashkiv, L.B. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunity* **31**, 539–550 (2009).
18. Zhang, X. *et al.* Interferon-gamma exacerbates dry eye-induced apoptosis in conjunctiva through dual apoptotic pathways. *Invest. Ophthalmol. Vis. Sci.* **52**, 6279–6285 (2011).
19. Kasper, M. Heterogeneity in the immunolocalization of cytokeratin specific monoclonal antibodies in the rat eye: evaluation of unusual epithelial tissue entities. *Histochemistry* **95**, 613–620 (1991).
20. Krenzer, K.L. & Freddo, T.F. Cytokeratin expression in normal human bulbar conjunctiva obtained by impression cytology. *Invest. Ophthalmol. Vis. Sci.* **38**, 142–152 (1997).
21. Rios, J.D., Zoukhri, D., Rawe, I.M., Hodges, R.R., Zieske, J.D. & Dartt, D.A. Immunolocalization of muscarinic and VIP receptor subtypes and their role in stimulating goblet cell secretion. *Invest. Ophthalmol. Vis. Sci.* **40**, 1102–1111 (1999).
22. Putney, J.W. Jr. Recent breakthroughs in the molecular mechanism of capacitative calcium entry (with thoughts on how we got here). *Cell Calcium* **42**, 103–110 (2007).
23. Kanno, H. *et al.* Cholinergic agonists transactivate EGFR and stimulate MAPK to induce goblet cell secretion. *Am. J. Physiol. Cell. Physiol* **284**, C988–C998 (2003).
24. Hodges, R.R., Bair, J.A., Carozza, R.B., Li, D., Shatos, M.A. & Dartt, D.A. Signaling pathways used by EGF to stimulate conjunctival goblet cell secretion. *Exp. Eye Res.* **103**, 99–113 (2012).
25. Hayashi, D., Li, D., Hayashi, C., Shatos, M., Hodges, R.R. & Dartt, D.A. Role of histamine and its receptor subtypes in stimulation of conjunctival goblet cell secretion. *Invest. Ophthalmol. Vis. Sci.* **53**, 2993–3003 (2012).
26. Ralph, R.A. Conjunctival goblet cell density in normal subjects and in dry eye syndromes. *Invest. Ophthalmol* **14**, 299–302 (1975).
27. Chen, Y., Chauhan, S.K., Saban, D.R., Sadrai, Z., Okanobo, A. & Dana, R. Interferon-gamma-secreting NK cells promote induction of dry eye disease. *J. Leukoc. Biol.* **89**, 965–972 (2011).
28. Tsubota, K. *et al.* Regulation of human leukocyte antigen expression in human conjunctival epithelium. *Invest. Ophthalmol. Vis. Sci.* **40**, 28–34 (1999).
29. Stern, M.E., Schaumburg, C.S. & Pflugfelder, S.C. Dry eye as a mucosal autoimmune disease. *Int. Rev. Immunol.* **32**, 19–41 (2013).
30. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). *Ocul. Surf.* **5**, 75–92 (2007).
31. Park, M.K., Petersen, O.H. & Tepikin, A.V. The endoplasmic reticulum as one continuous Ca(2 +) pool: visualization of rapid Ca(2 +) movements and equilibration. *EMBO J.* **19**, 5729–5739 (2000).
32. Smyth, J.T., Hwang, S.Y., Tomita, T., DeHaven, W.I., Mercer, J.C. & Putney, J.W. Activation and regulation of store-operated calcium entry. *J. Cell. Mol. Med.* **14**, 2337–2349 (2010).
33. Hogan, P.G., Lewis, R.S. & Rao, A. Molecular basis of calcium signaling in lymphocytes: STIM and ORAI. *Annu. Rev. Immunol.* **28**, 491–533 (2010).
34. Contreras-Ruiz, L., Ghosh-Mitra, A., Shatos, M.A., Dartt, D.A. & Masli, S. Modulation of conjunctival goblet cell function by inflammatory cytokines. *Mediators Inflamm.* **2013**, 636812 (2013).
35. Zhang, X., De Paiva, C.S., Su, Z., Volpe, E.A., Li, D.Q. & Pflugfelder, S.C. Topical interferon-gamma neutralization prevents conjunctival goblet cell loss in experimental murine dry eye. *Exp. Eye Res.* **118**, 117–124 (2014).
36. Smaili, S.S. *et al.* The role of calcium stores in apoptosis and autophagy. *Curr. Mol. Med.* **13**, 252–265 (2013).
37. Shatos, M.A., Rios, J.D., Tepavcevic, V., Kano, H., Hodges, R. & Dartt, D.A. Isolation, characterization, and propagation of rat conjunctival goblet cells in vitro. *Invest. Ophthalmol. Vis. Sci.* **42**, 1455–1464 (2001).
38. Horikawa, Y. *et al.* Activation of mitogen-activated protein kinase by cholinergic agonists and EGF in human compared with rat cultured conjunctival goblet cells. *Invest. Ophthalmol. Vis. Sci.* **44**, 2535–2544 (2003).
39. Rios, J.D., Ghinelli, E., Gu, J., Hodges, R.R. & Dartt, D.A. Role of neurotrophins and neurotrophin receptors in rat conjunctival goblet cell secretion and proliferation. *Invest. Ophthalmol. Vis. Sci.* **48**, 1543–1551 (2007).
40. Shatos, M.A. *et al.* Isolation and characterization of cultured human conjunctival goblet cells. *Invest. Ophthalmol. Vis. Sci.* **44**, 2477–2486 (2003).
41. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408 (2001).