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Investigation about T lymphocyte activation by human corneal cell types

Dissertation

Zur Erlangung des Grades eines Doktors der Medizin dem Fachbereich Medizin der Universität Hamburg vorgelegt von

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Contents

Abbreviations

APC	antigen presenting cell
$CD4^+$ T cells	helper T cells
$CD8^+$ T cells	cytotoxic T cells
CTL	cytotoxic T-lymphocyte
DTH	delayed-type hypersensitivity
EDTA	ethylenediamine tetraacetic acid
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
HCEC	human corneal endothelial cells
HLA	human leucocyte antigen
IL-2	interleukin 2
KO	knock out
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PHA	phytahematoagglutinin
SV40	Simian Virus 40
SV40 transfected HCEC	Simian Virus 40 human corneal endothelial cells
MHC	major histocompatibility complex
MST	median survival time
?-IFN	? interferon
RPE	retinal pigment epithelial
Th	T helper
TCR	T cell receptor

1. Aim of the study

Keratoplasty is a surgical procedure in which part or all of the cornea is replaced by healthy corneal tissue from a donor. In spite of keratoplasty is the most successful transplantation, cornea graft rejection is still the most frequent complication after corneal grafting and often leads to irreversible transplant failure. It has been concluded that corneal graft rejection, like other forms of organ transplantation, is a T cell mediated immune process. The development of an effective immune response required T cell activation by a first antigen as well as by a second costimulatory signal. The first signal is delivered when the T cell receptor (TCR) binds to the allogeneic peptide/HLA complex of the antigen presenting cells (APC), and the CD4 (in helper T cells) or CD8 (in cytotoxic T cells) co-receptors bind to a constant part of HLA (MHC) class-II or HLA (MHC) class-I molecules respectively. CD28-B7 (CD80/CD86) interactions provides possible "second signals" which are necessary for optimal T cell activation and IL-2 production. Other T cell surface molecules, such as CD40 ligand (CD154) also contribute to signal 2. Following signals 1 and 2, the T cell is fully activated so that the genes encoding lymphokines and lymphokine receptors are transcribed and translated.

In the Cornea Bank of the University Eye Hospital Hamburg-Eppendorf, methods for isolation and cultivation of human corneal epithelial cells and endothelial cells are established. These cultured cells should be examined for HLA expression. Additionally a method for isolation and cultivation of peripheral blood cells has to be established to investigate, whether co-culture of corneal cells and peripheral blood cells will lead to T cell activation. Furthermore, the expression of molecules with the potential to serve as second stimulator for T cell activation should be analyzed in the co-cultured cell system.

From the histology department, slides from corneas of patients who had cornea graft failures are available. This material offers the possibility to prove the relevance of the results obtained by the cell culture experiments. Presence of proteins involved in the T cell activation process in vitro will be analyzed by immunohistochemical staining of the corneas.

2. Introduction

2.1 The human eye and the cornea

The famous aphorism “the eye is the window of spirit”, not only emphasizes the importance of the eye, but also render prominent the presence of a window-pane, the cornea. The cornea, the conjuctiva, and the intervening transition area, known as the limbus, comprise the tissues of the ocular surface. The cornea is a tissue highly specialized to refract and transmit light. From histology aspect, the cornea consist of epithelium, stroma and endothelium. Although this avascular tissue seems simple in composition, it is extraordinarily regular and precisely arranged. All three layers have an uniform and consistent arrangement throughout the tissue in order to precisely bend and transmit light through to the lens, thence to the retina. (Fig. 21). The stroma is the middle connective tissue layer that is approximately 500 µm thick and comprises about 90 percent of the cornea. It is arranged in three clearly defined layers of extracellular matrix. These include, bordering the epithelium, the thin 8- to 10-µm Bowman´s layer; the middle lamellar stroma, and adjacent to the endothelium, the 8- to 12-µm Descemet´s membrane, the thickened basement membrane secreted by the corneal endothelium. The corneal endothelium is a single layered, low cuboidal endothelium. It plays a major role in maintaining stroma hydration through Na-K activated adenosine triphosphatase (ATPase) present in the basalateral membranes of the cells. Unlike the epithelium, the human endothelium is not a self-renewing cell layer. The number of endothelial cells decreases with age. As cells decrease in number, they become thinner and attenuated (Fig. 2.1) (Smolin and Thoft 1994).

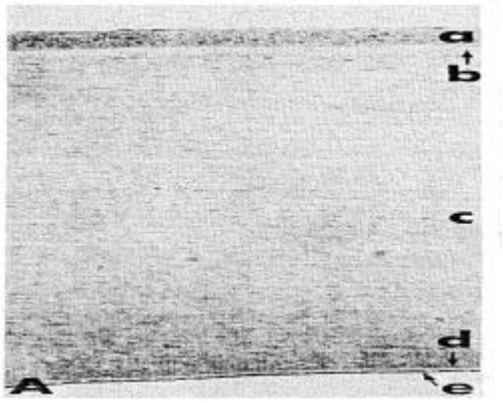


Fig. 2.1 Section through the central cornea. a=epithelium, b=Bowman´s layer, c=lamellar stroma, d=Descemet´s membrane, e=endothelium. [Smolin and Thoft (1994) The cornea-Scientific Foundations and Clinical Practice. Boston]

2.2 Corneal disease and corneal transplantation

There are many causes preventing normal morphology and function of the cornea, such as clouding of the cornea and abnormal corneal contour. For example: eye injuries that leave a dense white scar on the cornea, these injuries may include penetrating wounds from a sharp object, burns, or chemical contamination of the eye. Cataract or other eye surgeries can prompt corneal clouding. Corneal infection can also lead to scarring, the infection may be bacterial, viral, or fungal in origin. Various herpes viruses are known to cause such scarring. Keratoconus is the most typical disease for abnormal morphology. It may scar the center of the cornea or distort vision so severely that glasses or contact lenses are of little help. Corneal dystrophies may cause clouding, and some inherited diseases of the cornea results in abnormal function of the cornea. To solve these problems and retrieve a healthy cornea, the unique technology is corneal transplantation (keratoplasty).

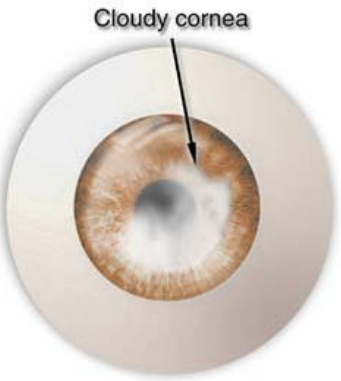
The history of corneal transplantation reaches back over 150 years. The first documented report of a successful penetrating keratoplasty in a human subject was performed by Zirm in 1906. As we enter the millennium, corneal transplantation remains the oldest, most common, and arguably, the most successful form of tissue transplantation (Niederkorn 2001). Neither HLA typing nor systemic immunosuppression (except in the case of high-risk individuals who have either received a previous corneal transplant or who have prevascularized graft beds) is performed routinely, it is remarkable that typical 2-year survival rates for initial grafts onto avascular graft beds are in excess of 90% (The Collaborative Corneal Transplantation Studies Research Group 1992). Keratoplasty is a surgical procedure in which part or all of the cornea is replaced by healthy corneal tissue from a donor. It can also be said it is a surgery to replace the clear window on the front of the eye (the cornea). Corneal transplant procedures may restore vision to otherwise blind eyes in some cases. It is divided into two forms: lamellar and penetrating keratoplasty. Lamellar keratoplasty: it is defined as removal and replacement of less than the total thickness of the cornea. As a rule, lamellar grafts tend to be relatively large (> 8 mm in diameter), and they replace tissue removed by deep stroma dissection. The host Descemet´s membrane and the endothelium are left intact and serve as the base onto which the donor tissue is laid. It is suitable for those corneal conditions in which the pathologic

changes are limited to anterior stromal and surface irregularities but in which the endothelium is healthy (Smolin and Thoft 1994). Most of the keratoplasty were mentioned to be penetrating keratoplasty. Tissue strengthening is the primary goal (tectonic) and increased visual acuity is the immediate aim. In the western world, the most frequent indication is keratoconus (followed eye Fuch´s endothelial dystrophies or secondary endothelial decompensation in the beginning of the 80th). Pseudophakic bullous keratopathy may account for about 17% of all corneal transplant procedures in the beginning of the south. Less frequent indications include corneal ulceration, corneal scars, herpes simplex and Varicella zoster viral infections leading to scarring, or congenital opacities of the cornea (Table 2.1).

author/country	number of patients	time	1. priority	2. priority	3. priority
Dandona/India 1997	1964		scarring 28,1 %	re-keratoplasty 17,1 %	ulcerating keratitis 12,2 %
Sharif KW/England 1993	3555	1971–1990	re-keratoplasty 40,8 %	keratoconus 17 %	herpetic keratitis 5 %
Frucht-Pery J/Israel 1997	1018	1961–1990	Keratoconus 21,8 %	re-keratoplasty 11,1 %	herpetic keratitis 9,3 %
Flowers CW, California/USA	1104	1989–1993	bullous keratopathy 24,8 %	re-keratoplasty 21,3 %	scarring / herpetic keratitis 11,1 %
Haamann, P/Denmark 1994	180	1984– 993	bullous keratopathy 28,3 %	keratitis 13,9 %	Fuchs dystrophy 13,9 %
De Cock R/Israel, 1994	416	198 –1992	keratoconus	keratitis/scarring	herpetic keratitis
Legeais, JM/Paris, France 2001	3736	1980–1999	keratoconus 28,8 %	herpetic keratitis 10,9 %	re-keratoplasty 9,9 %
Australian Corneal Graft Registry, 1993	3608	1985–1991	keratoconus 31 %	bullous keratopathy 31 %	re-keratoplasty 14 %
Dobbins KR, Middle West USA, 2000	4217	1982–1996	bullous keratopathy 31,5 %	Fuchs dystrophy 23,2 %	Keratoconus 11,4 %
Chen WL, Taiwan, 2001	770	1987–1999	scarring 27,9 %	re-keratoplasty 21,0 %	ulcerating keratitis 17,9 %
Hovding G, Norway, 1999			keratoconus		
Maeno A, Canada, 2000	6222	1964–1997	re-keratoplasty	keratoconus	bullous keratopathy
Graupner, M, Erlangen, Germany, 2000	207	1997–1999	keratoconus 44,9 %	Fuchs dystrophy 25,1 %	bullous keratopathy 16,9 %
Cursiefen C, Erlangen, Germany, 1998	2557	1992–1996	keratoconus 20,9 %	scarring/keratitis 20,4 %	bullous keratopathy 17 %
Lois N, Pennsylvania, USA, 1997	2186	1989–1995	bullous keratopathy 26,0 %	re-keratoplasty 17,8 %	Fuchs dystrophy 15,7 %
Chan CM, Singapore, 1997	327	1991–1995	bullous keratopathy 26,3 %	re-keratoplasty 11,9 %	corneal dystrophy 10,4 %

Table 2.1 Prevalence of diagnoses leading to perforating keratoplasty in dependence upon region and time. [Engelmann K, Fell A, Fankhauser II F (2002) Indikationsstellung zur Keratoplastik bei Keratokonus. Z Prakt Augenheilkd]

A



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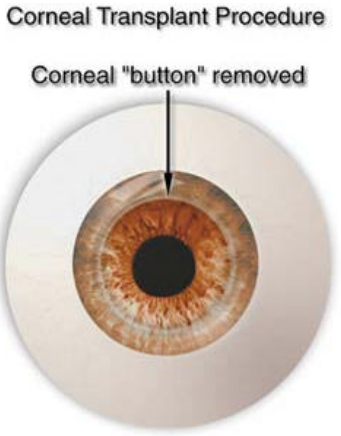

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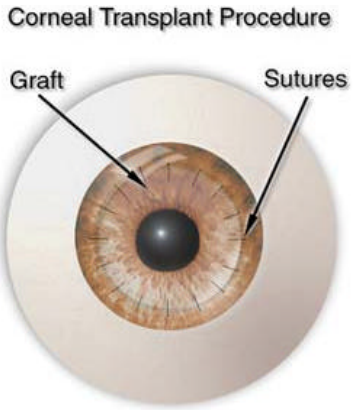

Fig 2.2 Cornea ulceration, herpes simplex, and damage or scar from disease or trauma lead to cornea opaque (A). Excision of recipient tissue: Pupils are usually miosed pre-op to avoid injuring the lens and causing cataract. The recipient cornea is then removed using a corneal trephine, Trephination is done with either the manual, motorized, or vacuum trephine. The donor button is ideally 0.5 mm larger than the planned recipient opening (In case if the donor button is trephined from the endothelial side). No over size, if both corneas are rephined from the epithelial side (B). The donor cornea is gently sewn into recipient bed, four cardinal interrupted sutures are applied at 12, 3, 6, 9 o´clock respectively. Interrupted or running sutures are then performed for final fixation (C). Taken from (www.eyemdlink.com/EyeProcedure.asp?EyeProcedureID=65).

2.3 Complications after transplantation- graft rejection

In spite of keratoplasty is the most successful transplantation, cornea graft rejection is still the most frequent complication after corneal grafting and often leads to irreversible transplant failure. It is defined as a specific process in which a graft, having been clear for at least several weeks (usually longer), suddenly succumbs to graft edema in conjunction with inflammatory signs. This process is immunologically mediated, which represents the end stage of the immunologic reaction that is no longer reversible. Many inferences about the immunobiology of corneal graft rejection have been based on clinical observations; however, confirmation of these hypotheses requires prospective studies under controlled settings. The prudent use of animal models has fostered analytic studies on the immunobiology of corneal allografts without the complicating and confounding effects of topical steroids that are typically used on most keratoplasty patients. Although animal models of penetrating keratoplasty have been in use for almost a half-century, until recently, progress in understanding the immune mechanisms of corneal graft rejection has been slow. Nevertheless, the widespread use of rodent models of orthotopic corneal transplantation has shed new light on the pathogenesis of corneal graft rejection. These findings indicate that prevention and therapy of allograft rejection is still the most challenging field of today´s keratoplasty. It is well known that T cells are critical to graft rejection. An immunohistochemical study by Williams et al. (1989), has shown that half the leucocytes detectable in corneal grafts are T lymphocytes, two-thirds bore MHC class II markers and one-fifth carried myeloid cell markers.

Rejection of organ and tissue allografts occurs because the mammalian genome contains several polymorphic loci that encode widely expressed tissue antigens. Persons who do not express a given allele at any of these loci recognize the protein encoded by that allele as foreign and mount a vigorous immune response that leads to graft rejection. The most important genes are clustered within the major histocompatibility complex (MHC), which in humans is known as the HLA complex. For many reasons, it is likely that the human leukocyte antigen (HLA) alloantigens are involved in corneal graft rejection. They are known to be the main targets for the immunological reactions leading to rejection of transplanted solid organs. There are two main classes of HLA molecule: HLA class I and class II. The function of the classical HLA class-I molecules,

which include HLA-A, B, C, is to present peptides to $CD8^+$ T cells (cytotoxic cells), whereas the function of HLA class- II molecules, HLA-DR, DQ and DP, is to present peptides to $CD4^+$ T cells (helper T cells) (Berry 1999). HLA class I antigens are expressed by the corneal epithelium, keratocytes and endothelium. HLA class II molecules are found on dendritic cells (Langerhans cells) in the superficial epithelium layers, and their expression is induced by inflammation due to infection, rejection and even the transplantation procedure itself (Whitsett and Stulting 1984; Fujikawa et al. 1982; Young et al. 1985; Tressler 1984). Their normal function is to bind fragments of antigenic peptides derived from invading microorganisms and present them to cells, which recognize them through their antigen receptors. This recognition step initiates T-cell-mediated immune responses.

2. 4 Components of the immune system related to graft rejection

Despite of almost 50 years of research using on keratoplasty, the precise immunologic mechanisms of corneal graft rejection remain a mystery. The seminal studies of Maumenee (1951) provided the first evidence that the immune system might contribute to corneal graft failure. In the late 1960s and mid-1970s, Khodadoust and Silverstein (1969, 1976) performed a series of studies using a rabbit model of keratoplasty demonstrating that corneal graft rejection was a cell-mediated phenomenon, could be adoptively transferred to naive hosts, all three layers of the corneal allograft were independently susceptible to immune rejection and rejection could be induced by adoptive transfer with immune lymphocytes. These findings led many investigators to conclude that corneal graft rejection, like other forms of organ transplantation, was a T cell mediated immune process. Two fundamental T cell-mediated effector mechanisms have been implicated in organ graft rejection: piecemeal necrosis of corneal cells by $CD8^+$ CTL (cytotoxic T lymphocyte) and DTH (delayed-type hypersensitivity) mediated by $CD4^+$ T cells.

Although many cells can participate in the process of cornea transplant rejection, only T lymphocytes appear to be absolutely required (Niederkorn 2001). Lymphocytes arise from stem cells in the bone marrow that divide to give rise to an expanding population of uncommitted *lymphocyte precursors*. In mammals, which have no bursa of Fabricius, lymphocyte precursors do not seem to require a special extramedullary environment to continue their development into B lymphocytes. They are believed to

complete their differentiation in the bone marrow. In both mammals and birds, other lymphocyte precursors are carried in the blood from the bone marrow to the thymus, where soluble factors produced by stromal cells induce their differentiation into *T lymphocytes*, which are capable of binding antigen and causing lysis of foreign cells. There are several kinds of T lymphocytes, but the principal categories are cytotoxic T-lymphocytes, which are the effector cells of cell-mediated immune responses, and helper T-lymphocytes, which participate in both humoral and cell-mediated responses (Leffell 1997).

2.4.1 Role of CTL in corneal graft rejection

The development of the rat model of penetrating keratoplasty by Williams and Coster in 1985, and the mouse model by She and coworkers in 1990, created new tools for examining the role of CTLs in the rejection of orthotopic corneal allografts. Studies in both the rat and mouse models of corneal transplantation demonstrated a correlation between the appearance of donor-specific CTLs and corneal graft rejection (Van der Veen et al. 1998; Minamoto et al. 1994; Ksander et al. 1996; Pleyer et al. 1995). Moreover, the role of lymphocytes in corneal graft rejection was supported by the appearance of the so-called "epithelial rejection line," which was characterized by a discrete zone of dead and dying epithelial cells surrounded by leukocytes, in front of which were apparently normal donor epithelium and behind which was a thin layer of dead donor epithelial cells (Khodadoust and Silverstein 1969). Classic CTL express the CD8 surface marker and use cytolytic proteins, called perforins, that perforate the cell membranes of target cells. Such piecemeal necrosis is consistent with the pattern of rejection one might observe if corneal graft rejection were mediated by CTLs, that is, CTL-mediated killing is contact dependent and occurs in a piecemeal fashion, and led many investigators to conclude that corneal graft rejection is mediated by CTL. Therefore, if corneal graft rejection were solely mediated by CTL, one would predict that corneal allograft rejection would be impaired or prevented in mice deficient in either of these molecules (Niederkorn 2001). However, studies using in vivo depletion of CD8+ T cells with monoclonal antibody failed to significantly enhance corneal graft survival in mice and thus cast doubt on the importance of conventional CTLs in corneal graft rejection. (He et al 1991) Recently, Hegde and Niederkorn (2000) took advantage of two different gene knockout mice to evaluate the role of perforin- and $CD8^+$ T-cell–

dependent mechanisms in the rejection of orthotopic corneal allografts, they found that corneal graft rejection occurs unabated in both CD8 knockout (KO) mice and in perforin KO mice, both of which are incapable of developing allospecific CTL responses, and concluded that CTLs do not play a role in the rejection of MHC and minor H mismatched corneal grafts. The ability of both perforin- as well as $CD8^+$ T-cell–deficient hosts to reject donor corneas as effectively as wild-type controls indicated that conventional CTLs are not essential for corneal allograft rejection.

2.4.2 Role of $CD4^+$ immune mechanisms of corneal graft rejection

In some previous studies, a close correlation between the development of DTH (delayed-type hypersensitivity) to donor histocompatibility antigens and corneal allograft rejection has been emphasized (Joo 1995; Sonoda and Streilein 1993). Classical DTH responses are mediated by a subset of T cells that express the CD4 surface determinant. Using rats and mice as experimental model, depletion of $CD4^+$ T cells in vivo with monoclonal antibody leads to a sharp reduction in corneal graft rejection. Moreover, it is unsuccessful for CD4 KO mice either to develop DTH responses to donor histocompatibility antigens or to reject their orthotopic corneal allografts (Niederkorn 2001).

Suppression of $CD4^+$ T cell dependent process seems to be able to improve corneal allograft survival if DTH were necessary for corneal graft rejection. The unique immune privilege of the anterior chamber was used to indicate this hypothesis. Investigation on rats, mice, and primates have displayed that antigens introduced into the anterior chamber of the eye generate a deviant immune response culminating in the antigen-specific downregulation of DTH. Anterior chamber inoculation of cells bearing donor histocompatibility antigens can significantly suppress the host´s DTH response to donor histocompatibility antigens, and remarkablely increase the corneal allograft survival in both rat and mouse models of penetrating keratoplasty (Niederkorn and Mellon 1996).

According to different functions and the cytokines, $CD4^+$ T cells can be further divided into two classes: Th1 and Th2 cells. Th1 cells produce interleukin-2 (IL-2) and the proinflammator cytokine interferon-? (IFN-?). DTH is a classic Th1-mediated lesion, which is characterized by the presence of a mononuclear infiltrate and a conspicuous absence of granulocytes. Otherwise, Th2 cells can produce a unique spectrum of cytokines, which includes IL-4, IL-5 and IL-10. Current dogma confirms

that DTH is a Th1 immune process mediated by $CD4^+$ T cells, which produce the proinflammatory cytokine IFN-?. Studies in both humans and experimental animals have found that rejected corneal allografts contain mononuclear infiltrates, including $CD4^+$T cells. Moreover, the Th1 cytokine (IFN-?) as one of the important cytokines can be expressed by the infiltrating inflammatory cells in rejecting rat coneal allografts.

Yamada et al. (1999) studies with mice genetically deficient in expression of CD4 or CD8 molecules were performd to determine which T cells are responsible for rejection of orthotopic corneal allografts in mice. Corneas grafted to CD8KO mice were rejected with an incidence and tempo indistinguishable from that in wild-type control animals. By contrast, MHC only, and minor-H-only incompatible corneal grafts survived indefinitely in eyes of CD4KO mice. Approximately 50% of corneal grafts that confronted CD4KO recipients with both MHC and minor H alloantigens experienced delayed rejection, whereas similar grafts in wild-type recipient were rejected acutely. The authors concluded that $CD8^+$ T cells played little or no role in corneal graft rejection and that MHC is exclusively mediated by $CD4^+$ T cells.

Similar study was performed by Tanaka et al in 2000 who showed that the time and tempo of corneal xenograft rejection is acute rejection of orthotopic corneal allografts. Acute rejection is mediated almost identical in normal mice compared with mice in which the µ chain of immunoglobulin had been disrupted. In the study by Tanaka et al., mice with a disrupted ß-2 microglobulin (ß2µ) gene also rejected guinea pig corneal grafts in an acute manner similar to normal mice. This finding strongly suggests that the acute phase of cell-mediated xenograft rejection in mice is neither mediated by $CD8^+$ cytotoxic T cells nor by NK T cells (both of which are depleted in ß2µ knockout mice). Alternatively, Tanaka et al. showed that mice deficient in $CD4^+$ T cells no bnger reject guinea pig corneal grafts acutely (MST, median survival time) 27 days, implying that mice reject corneal xenografts acutely, using xenoreactive $CD4^+$ T cells (Tanaka et al. 2000).

Mice with genetic or induced T–cell deficiency cannot reject grafts because they lack the cellular mechanisms to recognize an antigen as foreign. Duquesnoy et al. (1991) propagated and characterized lymphocytes from transplant biopsies. The technique of growing T-cells line from rejected allografts using recombinant interleukin-2 (IL-2) has enabled recognition of the cells involved in allograft rejection

(Wackenheim-Urlacher et al. 1995). Rejected corneas were invaded by a mixture of activated $CD4^+$ and $CD8^+$ T-cells, with one population being predominant. The development of an effective immune response required T cell activation by antigen and a second costimulatory signal (Janeway and Bottomly 1994). Immune responses may be divided into three phases: recognition, activation and effector mechanisms. The same is the case for alloimmune responses.

2.4.3 T cell allorecognition

T cells are able to recognize foreign (non-self) peptides in complexes with self-HLA (MHC) molecules. The peptide/HLA complex is recognized by the T cells by its specific TCR (T cell receptor). During the development from bone marrow stem cells, the maturing T cell population is able to generate a very large number of TCR variants, which are able to recognize an enormous variety of peptide/HLA complexes. During maturation in the thymus, however, only those T cells that are able to recognize peptides presented by self-HLA molecules are positively selected. T cells that generate TCRs that are only able to recognize peptides presented by foreign-HLA molecules are useless to the body and, are not selected; they die of neglect.

HLA molecules are general peptide receptors that do not distinguish between peptides derived from foreign or self-proteins. They therefore bind all peptides that, in form and charge, fit into their cleft. Positively selected T cells are therefore able to recognize self and non-self (foreign) peptides alike. T cells that are able to recognize self-peptides bound to self-HLA molecules are potentially autoreactive and this may be dangerous. They are therefore deleted during maturation in the thymus (negative selection) or are otherwise inactivated or silenced in the periphery.

Recipient T cells are able to perceive allogeneic donor cells and tissue in two different ways:

Indirect allorecognition: T cells may recognize foreign peptides derived from proteins of the transplant and presented by the self-HLA molecules of recipient antigen-presenting cells (APC).

Direct allorecognition: T cells directly recognize foreign (non-self) HLA molecules or (usually foreign) peptide/HLA complexes that are present on the transplanted cells.

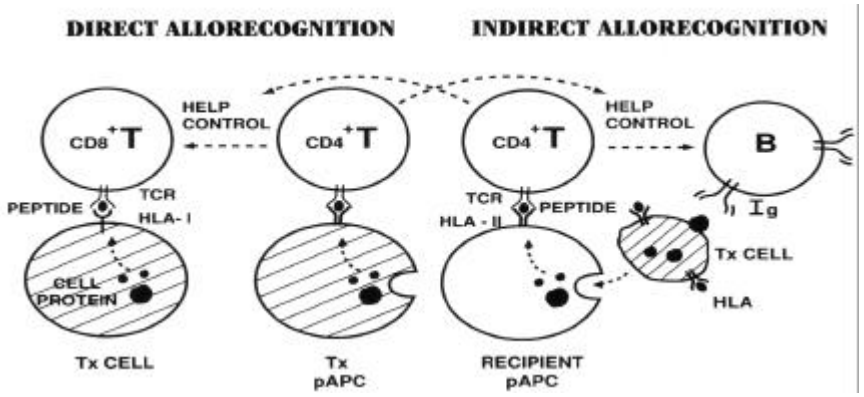

Fig. 2.3 An overview of indirect and direct allorecognition. $CD4^+$ T cells (*T*) involved in indirect recognition are able to recognize material from transplanted (*Tx*) cells (*cross-hatched*) after the transplanted cells-or fragments from them-are taken up, processed and displayed by a host professional antigen-presenting cells (PAPC). B cells (B) may perceive foreign molecules directly on the transplanted cell. $CD4^+$ T cells and $CD8^+$ T cells involved in direct allorecognition recognize the peptide/HLA complexes on transplanted cells. [Berry (1999) Transplantation pathology: a guide for practicing pathologists. Springer, 1999]

2.4.4 Mechanism of T cells activation

The two-signal model of T-cell activation is still valid after 30 years. (Bernard et al. 2002) It was originally proposed by (Bretscher and Cohn 1970) in an attempt to account for self-tolerance in the periphery. The model in its present form assumes that T cells require two distinct but synergistive signals to be activated by antigen-presenting cells (APC). The first signal is delivered when the T cell receptor (TCR) binds with sufficient avidity to the allogeneic peptide/HLA complex of the antigen presenting cells (APC), and the CD4 (in helper T cells) or CD8 (in cytotoxic T cells) co- receptors bind to a constant part of HLA (MHC) class-II or HLA (MHC) class-I molecules respectively. An activation signal 1 is transduced into the T cell, it is responsible for the specificity of the immune response. Signal 1 is necessary, but not sufficient to activate T cells. Other signals: collectively called signal 2, or costimulatory, are not antigen-specific, but are also necessary for activation. Many T-cell molecules may serve as receptors for costimulatory signals; the CD28 molecule is the best characterized of these molecules. CD28 has two known ligands, B7-1 (CD80) and B7-2 (CD86), both of which are expressed primarily on activated antigen-presenting cells. Other T cell surface molecules, such as CD40 ligand (CD154), CD2, LFA1 and ICAM 1, contribute to signal 2. Upon antigen presentation, the receptor-ligand pairs CD28-B7 (CD80/CD86) and CD40-CD154 are essential for the initiation and amplification of T-cell-dependent

immune responses. CD28 B7 (CD80/CD86) interactions provide "second signals" necessary for optimal T cell activation and IL-2 production, whereas CD40-CD154 signals co-stimulate B-cell, macrophage, endothelial cell and T-cell activation (Larsen 1996). Following signals 1 and 2, the T cell is fully activated so that the genes encoding lymphokines and lymphokine receptors are transcribed and translated.

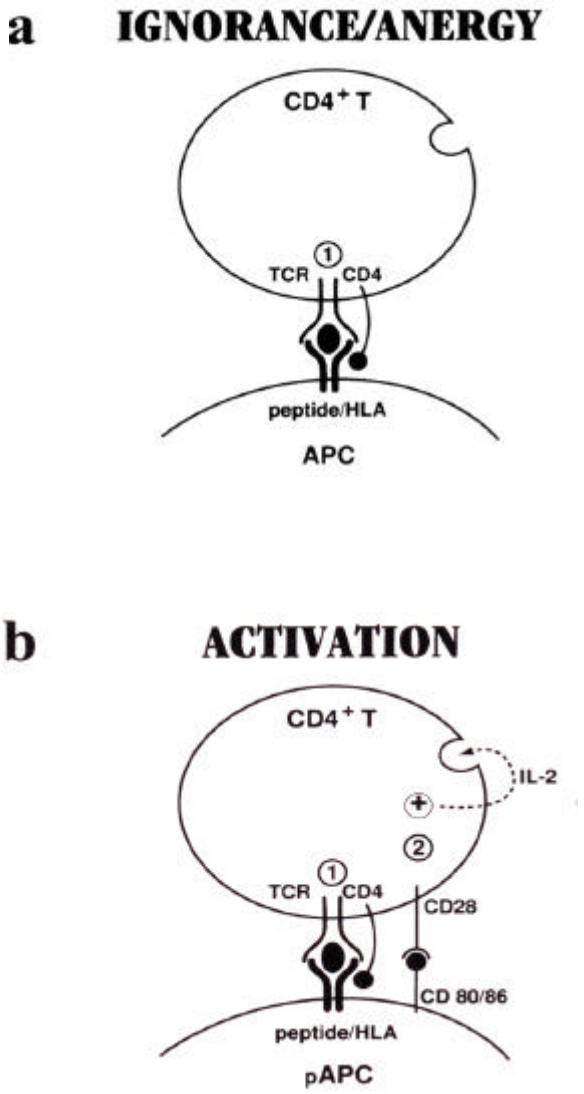

Fig. 2.4 $CD4^+$ T cells, which bind only the peptide/human leucocyte antigen (*HLA*) complex and not other accessory molecules, will get only signal-1, will ignore the foreign peptide/HLA or will be made anergic (a). $CD4^+$ T cells which, by their T cell receptors (*TCR*), recognize the peptide/HLA complex and with their CD28 molecule bind to the CD80 and/or CD86 molecules, get both signal-1 and signal-2. This will lead to a positive (+) signal for synthesis of interleukin-2 (IL-2), which binds to the IL-2 receptor. Consequently, the T cell will be fully activated and will proliferate. *PAPC*, professional antigen-presenting cells (b). [Berry (1999) Transplantation pathology : a guide for practicing pathologists. Springer, 1999]

Optimal and sustained T cell responses require costimulatory signals delivered through one or more receptors on the surface of T cells (Janeway and Bottomly 1994). T cell costimulaiton by B7 molecules plays an important role in the regulation of alloimmune responses. The CD28-B7 pathway is critical to T-cell costimulatory activation in alloimmune responses. There is increasing evidence that ongoing T-cell recognition of alloantigen and activation are key mediators of chronic allograft rejection (Womer et al. 2000). B7 blockade by CTLA4 immunoglobulin (Ig), which binds B7-1 and B7-2, prevents acute allograft rejection and induces donor-specific tolerance in several experimental transplant models (Pearson et al, 1996, 1997). In some models, B7 blockade also prevents the development of chronic rejection (Sayegh et al. 1997; Kim et al. 2001). Several studies have been undertaken to address the mechanisms of tolerance by blocking CD28 mediated costimualtion. Depending upon the model examined, anergy, deletion, and suppression have all been implicated (Bluestone 1995; Sayegh et al. 1995; Tran et al. 1997; Judge et al. 1996). Blockade of CTLA-4 or B7-1 significantly accelerated graft rejection. In contrast, B7-2 blockade significantly prolonged allograft survival, and unexpectedly, reversed the acceleration of graft rejection caused by CTLA-4 blockade (Yamada et al. 2001). Blocking the interaction of the CD28 costimulatory receptor with its ligands, CD80 and CD86, inhibits in vivo immune responses, such as allograft rejection, and in some instances induces tolerance (Thomas et al. 1999). When Anti-CD80 and anti-CD86 monoclonal antibodies were administered after orthotopic corneal allograft in mice, it showed prolonging corneal allograft survival (Kagaya et al. 2002). The ability of CD28 to mediate signaling of T cells has been extensively demonstrated in a number of experimental systems, but evidence has also been reported for signaling of B cells through CD80/86 (Hirokawa et al. 1996; Jeannin et al. 1997; Kasprowicz et al. 2000; Suvas et al. 2002). Thus, a role for CD80/86 signaling in costimulus-dependent activation of B cells is plausible but has not been directly assessed (Joanne et al. 2003).

It is well established that the CD154: CD40 pathway is important in the generation of cell-mediated immunity. Blocking CD154: CD40 led to enhanced tolerance in chemically induced diabetic mice (Parker et al. 1995). In experimental transplantation, blockade of CD40-CD40 ligand interactions has proved effective in permitting long term graft survival, disruption of the CD154:CD40 pathway conferred increased

acceptance of cardiac allografts (Larsen et al. 1996; Hancock et al. 1996; Niimi et al. 1998; Shepherd and Kerkvliet 1999), treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates (Kirk et al. 1999), and suggest that the generation of allograft immunity is dependent on the interaction of CD154 with CD40. Likewise, Van ED et al. (1995), showed that T cells activated in the absence of CD40 were unable to help normal B cells to undergo Ig class switching or germinal center formation. Recently, blockade of CD40: CD154 pathway has been approved for clinical evaluation, Jones et al. (2000) demonstrated that the administration of an anti-CD154 mAb prevent CD4+ T cell mediated rejection, but CD8+ T cells remained fully functional. Qian and Dana (2002) further confirmed that the use of systemic anti-CD154 therapy in the mouse model of corneal transplantation promote corneal allograft survival (Qian et al. 2001).

2.4.5 Regulation of HLA class II and CD40 expression by ?-interferon

For many reasons, it is likely that the human leukocyte antigen (HLA) alloantigens are involved in corneal graft rejection. They are known to be the main targets for the immunological reactions leading to rejection of transplanted organs (Bertelmann et al. 2002). The HLA class II antigens, are the products of the immune response genes and play a major regulatory role in interaction between immunocytes. Originally, the class II antigens were thought to be expressed only on the surface of the cells of the immune system. However, in many cell types that do not normally express the HLA class II antigens, it has been shown that during cellular immune responses such as those found in autoimmune diseases or allograft rejection, these cells express the class II antigens at inflammatory sites (Hall et al. 1984; Most et al. 1986; Volc-Platzen et al. 1984; Chan et al. 1986). In addition, the expression of class II antigens can be induced by interferon gamma (?-interferon) in vitro.

In the cornea, it has been shown that class II antigen expression appears to be limited normally to Langerhans cells and endothelial cells lining limbal blood vessels. However, HLA antigen are expressed on corneal cells after allograft rejection. ?-interferon can induce the expression of HLA-DR antigen on epithelial cells, stromal fibroblasts, and endothelial cells in vitro. Young et al. 1985; Dreizen et al. 1988; Donnely et al. 1988; EL-Asrar et al. (1989) induced HLA-DR expression on human cornea epithelium, using excised scleral margin pieces. And Iwata et al. (1992)

successfully induced HLA class II antigen expression on human cornea cells in culture by ?-interferon.

The same method used on RPE cells, Gabrielian et al. (1994), Osusky et al. (1997) confirmed retinal pigment epithelial cells induced to express HLA-DR by incubation with interferon gamma, suggested that MHC class II expressing RPE cells could contribute to immune and inflammatory activity in the eye by presenting antigens to T lymphocytes. Willermain et al. (2000) demonstrated ?-interferon activated human retinal pigment epithelial cells expressed CD40, but not CD80 or CD86, and they did not stimulate allogeneic resting T cells and downregulated phytohemagglutinin (PHA)-activated allogeneic T cells via a cell-to-cell contact dependent mechanism, but inhibit T-cell proliferation, partly through induction of apoptosis.

2.5 Effector mechanisms in allograft rejection

When alloreactive CD4+ T cells are activated on recognition of foreign peptide HLA class-II complexes, they proliferate and, at the same time, secrete a set of lymphokines. $CD4^+$ T cells may differentiate into T helper 1 (Th1) cells or T helper-2 (Th2) The alloreactive T cell response seems to be mainly a Th1 response, with secretion of IL-2 and interferon gamma (γ–IFN). IL-2 helps sustain proliferation of $CD4^+$ T cells that may differentiate into T helper 1 (Th1) cells or T helper-2 (Th2) and $CD8^+$ T cells that, at the same time, have recognized foreign peptide/HLA class-I complexes. If NK cells fail to recognize their self-HLA molecules on the grafted cells, they are no longer inhibited and may kill the target cells. ?-IFN both enhances the killer potential of $CD8^+$T and NK cells (nature killer cells) and, meanwhile, activates monocytes/macrophages. Help from alloreactive $CD4^+$ Th2 cells is required for activation, proliferation and differentiation of alloreactive HLA-specific B cells into Plasma cells that -depending on the HLA disparity between donor and recipient -secrete antibodies specific for donor HLA class-I or class-II molecules.

In concert, alloreactive T cells, NK cells and monocytes attack and destroy the foreign cells, both by direct cell-to-cell contact killing and by means of the cytokines secreted by these effector cells. Direct cell-to-cell contact T cell killing is mediated through two different pathways. One is the expression of Fas L by activated T cells, which binds to Fas (CD95) on the target cell and, thereby, initiates apoptosis of the target cells. Alternatively, $CD8^+$ T or NK cells may kill target cells through the release

of granules containing perforin and granzyme B. Perforin will, at high concentrations, lead to necrotic cell death by forming lytic pores in the cell membrane, or it may, at sublytic concentrations, enhance the transport of granzyme B into the cell where it initiates an apoptotic program similar to that mediated by Fas. During acute rejection, $CD4^+$ T cells, $CD8^+$T cells, NK cells, B cells and monocytes /macrophages are all present among graft-infiltrating cells, and cytotoxic effector and delayed-type hypersensitivity are the main mechanisms responsible for the destruction of the graft.

If HLA antibodies are produced after transplantation, they may -in concert with T and NK cells -contribute to rejection by activating complement or by enhancing the activity of monocytes and NK cells, via enhanced Fc receptor binding to target cells. By binding directly to HLA molecules of the endothelial cells of the transplant and activating complement, preformed HLA class-II specific antibodies do not usually cause hyperacute rejection, probably because endothelial cells express low levels of HLA class-II molecules (Berry 1999).

3. Material and Methods

3.1 Cell culture

3.1.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood mononuclear cells (PBMC) were separated from fresh blood of healthy volunteer (The blood bank, University Hospital in Hamburg-Eppendorf (UKE), Germany) by HISTOPAQUE-1077 (SIGMA DIAGNOSTICS; INC; USA) density-gradient. Approximately 15 ml blood was collected from normal human volunteer donors in accordance with the ethical standards of the Hamburg-Eppendorf University Hospital Institutional Review Board on human experimentation. 10 ml HISTOPAQUE-1077 was added into centrifuge tube, and 10ml fresh blood was laid onto the HISTOPAQUE-1077. The gradient was centrifuged at 400 *g* for 30 minutes. After centrifugation, the opaque interface was transfered into a clean conical centrifuge tube, and 10 ml Isotonic Phosphate Buffered Saline Solution was added. The cell suspension was centrifuged at 250 *g* for 10 minutes. This washing step was repeated twice. Cell pellet was resuspended in RPMI 1640 medium with 2mM L-glutamine, 1mM Hepes solution (GIBCO TM Invitrogen, Karlsruhe, Germany), supplemented with 10% FCS (Fetal Calf Serum, GIBCO TM Invitrogen, Karlsruhe, Germany) and 100 unit/ml penicillin-streptomycin (GIBCO TM Invitrogen, Karlsruhe, Germany). A cell count and trypan blue exclusion viability assay was performed. A new blood donor was used for each repetitive experiment.

3.1.2 Human Corneal Epithelial cells

Human limbal epithelial cells were isolated from donor corneas unsuitable for keratoplasty or from the remaining scleral rims of donor corneas after trephination. Donor corneas/scleral rims were put into 24-well culture dishes with 0.5 ml culture medium (Pellegrini et al. 1997). Epithelial cells of the limbal area were scrapped by means of a hockey knife. The corneas/ scleral rims were removed and the cells were cultured at 37°C and 5% CO_2. Isolated limbal epithelial cells were incubated with epithelium medium, consisting of Ham´s F12 and Dulbecco´s modified Eagle´s medium (DMEM; 1:1, GIBCO TM Invitrogen, Karlsruhe, Germany), containing 10ng/ml mouse epidermal growth factor (Seromed Biochrome, Berlin), 5ug/ml bovine insulin (Sigma), 0.1ug/ml cholera toxin (Sigma), 5mg/l transferrin (Sigma), 0.18mmol/l adenine (Sigma), 0.4mg/l hydrocortison (Sigma), 2nmol/l 3, 3, 5 triiodin-thyronine (Sigma),

4mmol/l glutamine (Biochrom KG, Berlin), 40 µg/mL gentamicin (GIBCO TM Invitrogen, Karlsruhe, Germany), amphotericin B (Biochrom KG, Berlin) and 10% fetal calf serum (FCS, GIBCO TM Invitrogen, Karlsruhe, Germany), in 24-well culture cluster (Costar, Coring Incorporated, U.S.A). The medium was changed twice a week.

3.1.3 Primary human corneal endothelial cells

Human donor corneas were prepared for organ culture as described previously (Böhnke, 1991). The corneas used in these experiments were considered unsuitable for corneal transplantation purpose due to low endothelial cell density.

Corneas were removed from postmortem human eyes. The donor age ranged from 30 to 70 years. Corneas were excised and stored in MEM medium supplemented with 10% FCS at 37°C up to 3 months. Human corneas were cultured in MEM supplemented with 2% FCS, 6% dextran, and penicillin (100 E/ml)/streptomycin (100 µg/ml) (Seromed Biochrom, Germany) 24 hours before denuded of their endothelium.

The corneas were transferred to a plastic dish with the epithelium turned downwards. The endothelium was covered with a few drops of collagenase IV (5mg/ml). Corneas were incubated at 37°C for 90 minutes. For isolation of HCEC the endothelium was rinsed with 20 ml of IF (a 1:1 mixture of Iscove´s Dulbecco´s modified Eagle´s medium and Ham´s F12) using a sterile syringe combined with a thin needle (No.14, 0.65×30mm). Isolated cells were separated by centrifugation at 900 *g* for 10 minutes, resuspended in HCEC growth medium as described (Engelmann and Friedl 1989) and seeded in one well of a 24-well plate. All culture dished used for HCEC were coated using a mixture of laminin (GIBCO TM Invitrogen, Karlsruhe, Germany) and chondroitin sulfate (0.5%, Sigma) in culture medium. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For passaging the cells, medium was removed from the confluent primary cultures, cells were rinsed once with PBS and further treated with an 0.25% trypsin and 0.5% ethylenediamine tetraacetic acid (EDTA, Sigma) solution for approximately 5 minutes.

3.1.4 SV40 transfected human corneal endothelial cells

Most of the experiment were performed using the SV40 transformed HCEC-cell line, which was established in the laboratory as described elsewhere (Bednarz et al. 2000). The immortalized cells were cultured in medium-F99 [a 1:1 mixture of Ham´s F12 (GIBCO TM Invitrogen, Karlsruhe, Germany) and M199 (GIBCO TM Invitrogen,

Karlsruhe, Germany)] supplemented with 5% fetal calf serum. They were cultivated in 25 cm^2 costar flasks (Corning Incorporated, U. S. A) and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

3.1.5 Human retinal pigment epithelial cell isolation and cultivation

Media and supplements were purchased as powders and dissolved with ultrapure water (Millipore Milli Quf plus filtration unit) according to the manufacturer´s instructions. Collagenase type I and IV (Sigma, Germany) were dissolved in basal medium F99, a 1:2 mixture of Ham´s F12 and medium 199 (GIBCO TM Invitrogen, Karlsruhe, Germany). All media were supplemented with 50 µg gentamycin/ml (GIBCO TM Invitrogen, Karlsruhe, Germany) and 2.5 µg amphotericin B /ml (Seromed Biochrom, Germany). For coating of culture dishes a 0.1% (w/v) gelatin (Merck, Germany) solution was used.

Human donor eyes were obtained from the Institutes of Pathology and Forensic Medicine of the University Hospital in Hamburg Eppendorf (UKE). Twelve donors were selected. Donor age ranged from 24 to 65 years (45 ±19.2 years), and postmortem times ranged from 12 to 41 h (23.0±8.2h). The cornea was excised from each eye ball. Then 3~5mm of the sclera, iris, lens and vitreous body, retina were removed by mechanical method. The remaining chorioid and attaching RPE (retinal pigment epithelium) were detached from sclera.The prepared choroid was washed in phosphate buffered saline (PBS) and incubated in 2 ml collagenase IA+IV (1+1)-mixture (concentration: 0.5 mg/ml; each type 0.25 mg/ml, Sigma) for 1-16 hours at 37°C and 5% CO_2. For coating, 0.5 ml of a 0.1% (w/v) gelatin (Merck, Germany) solution was added to every well of a 24-culture plate. The plate was incubated for 30 minutes. After removing the gelatin solution, the culture wells were washed with PBS. The cell suspension was separated from the choroid after incubation with a collagenase IA+IV (1+1)-mixture for 16 hours by centrifugation at 100 *g* for 5 minutes at room temperature. The cell pellet was resuspended in RPE-medium, and the cell suspension was distributed into the coated 24 well dish. Incubation was performed for 24 hours at 37°C and 5% CO_2 humidified environment. Then the medium of the culture wells was aspirated and the cells were washed with PBS. Finally 1 ml of RPE-medium was supplied to each culture well. The medium was changed every 2-3 days. RPE cells were incubated at 37°C and 5% CO_2 humidified incubator.

3.2 Preparation of chamber slides

Cultured human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE), were seeded on 25 cm^2 culture flasks, as described before. They were treated with 0.25% trypsin and 0.5% ethylenediamine tetraacetic acid (EDTA, Sigma) to single cell suspension. Cells were counted by means of a Coulter Counter (COULTER Electronics GmbH, Krefeld). 2×10^4 cells were seeded into the wells of a chamber slides (Nalge Nunc International). Parts of the cells were supplied with γ-IFN (1000 U/ml). After three days, the cells became confluent. One part of the cells cultured on chamber slides was washed twice in PBS. The cells were fixed with 70% ethanol in 50 mmol glycin buffer or 10% formalin. They were used for indirect immunohistochemical staining by mouse anti-human monoclonal antibody of HLA-DP, DQ, DR, CD40, CD80, CD86, CD154. The cells of the remaining chamber slides were washed twice in PBS and fresh purified PBMCs were directly added (3×10^5 cells /well). All cocultures were maintained in RPMI 1640 medium supplemented with 10% FCS, unless otherwise noted and incubated in 5% CO_2 at 37°C for two days. PBMCs cell suspension was poured out, and the cells attaching on the chamber slides were fixed with 70% ethanol in 50 mmol glycin buffer or 10% formalin. The fixed cells were used for indirect immunohistochemical staining by mouse anti-human monoclonal antibody against HLA-DP, DQ, DR, CD40, CD80, CD86 and CD154.

3.3 FACS Scan Analysis

3.3.1 Cell Preparation for FACS

Cell culture and preparation method were described above. After treatment with 0.25% trypsin and 0.5% ethylenediamine tetraacetic acid (EDTA, Sigma), the cells were counted and diluted to a concentration of 2×10^4/ml. 1 ml cell suspension of human corneal epithelial cells, human corneal endothelial cells, SV40 transfected human corneal endothelial cells and retinal pigment epithelial cells were delivered onto 12-well culture well. 1000 U γ-IFN was supplied into one well, was not supplied anything in another well. The cells were incubated in humidified 37°C, 5% CO_2 atmosphere. Medium was changed twice a week. Three days later, the cells became confluent. All the cells were then washed with PBS twice and fresh purified PBMCs were directly added (1×10^6) into all culture wells, including the cells stimulated with or without γ-

IFN. All cocultures were maintained in RPMI 1640 supplemented with 10% FCS medium, unless otherwise noted and incubated in humidified 5% CO_2, 37°C atmosphere. After cocultured for two days, the cocultured cell suspension was harvested for FACS analysis. FACS scan analysis was used on lymphocytes harvested after sufficient propagation. Two-color analysis was performed by means of FACS analyzer (Becton-Dickinson FACS-Calibur).

3.3.2 FACS analysis procedure

Following coculture for 2 days, the PBMCs were carefully removed via pipette from coculture well, avoiding touch sticking cells on the bottom. It was not possible to get all the PBMCs out of the coculture due to adhesion between the two types of cells. A recovery percentage was estimated to be not less than 75% (number of PBMCs recovered from coculture in comparison the number of PBMCs recovered from single culture). The cells were washed with PBS, then anti-$CD3^{FITC}$ (PharMingen International) and anti-$CD69^{PE}$ (PharMingen International), or isotype control antibody were added, followed by incubation at 4°C for 20 minutes. Then 1 ml phosphate buffered saline (PBS) was added to each sample, following centrifugation at 100 *g* for 10 minutes and removal of supernatants. Pellets were resuspended in 500 µl PBS, and propidium iodide (Sigma Chemical Co) was added to the samples 10 minutes before FC analysis, at a final concentration of 1 mg/ml, and analyzed within 4 hours. The FC instrument (FACScalibur, Becton Dickinson) was set using Auto comp software (Becton Dickinson) in conjunction with Calibrites (Becton Dickinson), or by an in-house instrument setting used for analyzing triple immunofluorescence stained samples. For each sample, 5000-10000 cells were acquired and saved as list mode data using CellQuest software (Becton Dickinson). Scatter regions for gating of lymphocytes and for monocytes based on their morphologic features (forward scatter for size) and staining by propidium iodide were initially optimized and thereafter used for all FC analysis (Fig. 4.3). The lymphocyte region was set to exclude very large lymphoblasts as these cells frequently were non-viable (shown by PI uptake) with increased autofluorescence. A few activated lymphoblasts were contained in the monocyte gate (see Fig. 4.4).

The expression of MHC class II (HLA-DP, DQ, DR), CD3, CD69 molecules on the cells was quantified by flow cytometry analysis with fluorescein isothiocyanate (FITC)

-or phycoerythrin (PE) -conjugated specific mouse monoclonal antibodies and control isotypes (all from Becton-Dickinson, Mountain View, CA). Cells were prepared according to standard prodedures. Briefly, 500,000 cells were washed and incubated for 20 minutes at 4°C in phosphate-buffered saline containing 0.1% NaN_3, 1% bovine serum albumin, and 10% human serum, to inhibit subsequent nonspecific labeling after antibody binding to fragment crystalline receptors (FcR). The cells were then incubated with saturating amounts (1µg/10^6 cells) of FITC-or PE-conjugated antibodies directed against specific surface antigens, for 30 minutes at 4°C, in the dark. Cells were then washed and resuspended in staining buffer before being analyzed using a FACScan flow cytometer and the Cellquest software (Becton-Dickinson).

3.4 Immunohistochemical staining procedure

3.4.1 Corneal samples

A total of 15 diseased corneas from patients undergoing penetrating keratoplasty were obatained at the Department of Ophthalmology, University Hospital in Hamburg Eppendorf (UKE), Germany. In the cases, 9 corneas were diagnosed as shown (Table 3.1). Age arranged from 54 to 82 years, average age: 68.6±9.6 years, male 3, female 6. Other 5 patients performed as control samples included keratoconus corneas. Age arranged from 22 to 33 years, average age: 27.5± 4.5 years, male 2, female 3. All the Human corneas were obtained by penetrating keratoplasty and sent to our Ophthalmic Histology Laboratory, fixed in 10% formalin, embedded in paraffin block, and cut into 3 µm sections.

Sex	Date of birth	The first diagnosis	Previous KP time	The second diagnosis	Current KP time
Female	04.04.1935	Cornea guttata by endothelial dystrophy	2000	Transplant decompensation	2000
Female	05.05.1924	Cornea decompensation after cataract surgery	1992	Transplant decompensation	2002
Female	28.10.1933	Sencondary endothelium atrophy	2001	Transplant decompensation, transplant ulcus	2002
Female	16.12.1942	Measles keratitis	1970	Transplant decompensation	2002
Female	08.09.1924	Chemical burn	1982	Transplant decompensation, vascularisation	1988
Female	21.05.1921	Cornea decompensation after phacoemulsification and posterior chamber lens implantation	2000	Transplant decompensation	2001
Male	05.07.1948	Corneal lattice dystrophy	1985	Transplant decompensation	2001
Male	03.04.1920	Transplant decompensation after keratoplasty after cataract surgery and posterior chamber lens implantation	1999	Transplant decompensation	2002
Male	28.02.1937	Chemical burn	1999	Transplant decompensation	2002

Table 3.1 Patients suffered from corneal transplant rejection. KP: keratoplasty

3.4.2 Cultured cells

Cultured human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE), which had been cocultivated with PBMCs were fixed by 70% ethanol in 50 mmol glycin buffer or 10% formalin on chamber slides. Indirect immunohistochemical staining procedure was as described below by monoclonal antibody of HLA-DP, DQ, DR (DAKO, Denmark); CD40 (DPC Biermann); CD154 (PharMingen International); CD80 (Ancell Corporation, U. S. A); CD86 (DAKO, Denmark).

3.4.3 Immunohistochemical staining procedure

After deparaffinization and rehydration, corneal sections were incubated with Protein Block Serum-Free (DAKO, Denmark) 10 minutes. Then were incubated respectively with 1:100 diluted mouse primary CD4 monoclonal antibody (Dianova) 60 minutes; CD8, HLA-DP, DQ, DR (DAKO, Denmark); CD40 (DPC Biermann); CD154 (PharMingen International) respectively 10 minutes. Followed by sequential 10 minutes incubations with biotinylated link antibody, slides were rinsed for five minutes with wash solution three times. Alkaline phosphatase-labelled streptavidin (DAKO, Denmark) was supplied and incubated for 10 minutes, slides were rinsed as before. Staining was completed after incubation with substrate-chromogen solution (DAKO, Denmark) 10 minutes. After the slides were rinsed, counterstaining was accomplished by hematoxylin. The sections were dipped into ammonia water, then specimens were mounted in an aqueous-based medium (AQUATEX, MERCK, Germany). Sections were photographed with a digital microscope with camera (Olympus U-LH 100 HG, Olympus Optical Co. Ltd. Japan)

4. Results

In this chapter, the results are presented in three parts. The first part is concerned with expression of cell surface molecules on human corneal epithelial cells, human corneal endothelial cells (HCEC), SV40 transfected human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE), including HLA-DP, -DQ, -DR, and the CD40 molecule. The second part is involved in the immunological characterization of cocultured cells: human corneal epithelial cells, HCEC, SV40 transfected HCEC and RPE cocultured with peripheral blood mononuclear cells (PBMCs). The third part deals with immunohistochemical analyses of tissues sections from corneal transplantation rejections.

4.1 Human corneal and RPE cells

4.1.1 Expression of HLA-DP, DQ, DR

Cultured human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE), were cultivated as described in the material and method section. Part of the cultures were supplemented with γ-IFN (1000 U/ml). After three days, the cells became confluent, and immunohistochemical staining using mouse anti-human monoclonal antibody was performed. Cultured human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE), were analyzed respectively, regarding to expression of HLA antigens. There was no staining on HCEC and SV40 transfected HCEC. Human retinal pigment epithelial cells (RPE) showed a low staining (Fig 4.1). Only less than 5% of the cells seemed to exhibit HLA expression. In contrast, staining was very frequent for cultures of human corneal epithelial cells (more than 50% of the cells displaying HLA antigens). Additionally, HLA expression was analyzed after γ-IFN treatment. HLA class II antigen expression was clearly responsive to γ-IFN, all the cell types showed upregulation of HLA class II antigen expression. After γ-IFN stimulation, among these cells, nearly 100% positive staining were shown by RPE and human corneal epithelial cells, more than 95% for human corneal endothelial cells (HCEC), but only less than 25% for immortalized human corneal endothelial cells (SV40 transfected HCEC). In most of these samples,

positive staining could be observed either on the cell surface or in cytoplasm. The results are summarized in Table 4.1.

	Human corneal epithelial cells	**HCEC**	**SV40 transfected HCEC**	**Human RPE**
-γ-IFN	+++	-	-	+
+γ-IFN	++++	+++	+	++++

Table 4.1: Immunohistochemical detection of HLA-DP, DQ, DR (human leukocyte antigen) expression on cultured corneal cell types and retinal cells. Cells were treated or not with γ-IFN (γ-interferon) 1000 U/ml. HCEC: human corneal endothelial cells; RPE: retinal pigment epithelial cells; SV40 transfected HCEC: immortalized human corneal endothelial cells. Relative staining intensity was indicated by: - (negative), + (weak, <25%), ++ (intermediate, 25~50%), +++ (strong, 50~75%), ++++ (very strong, 75~100%).

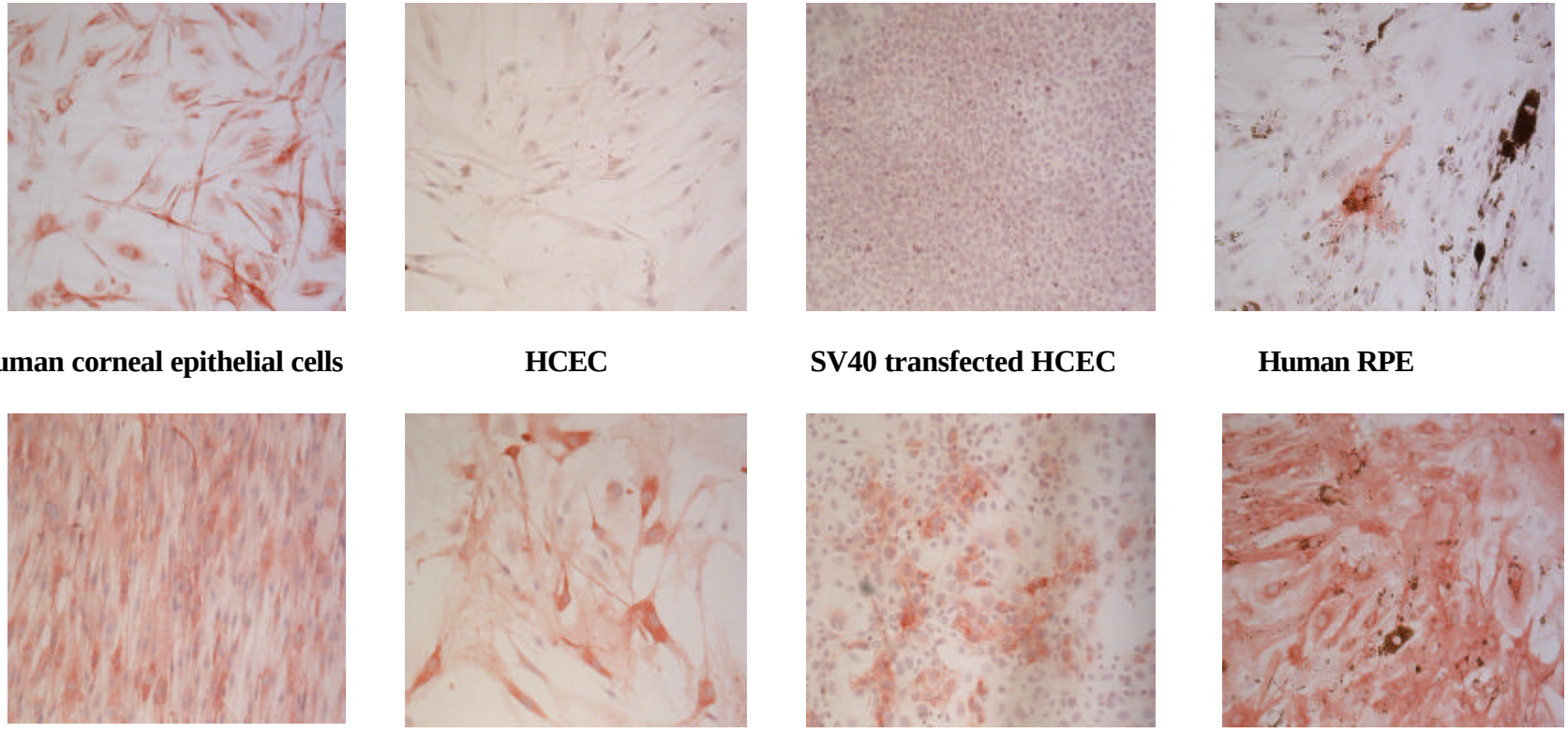

Fig. 4.1: Expression of HLA class II molecule on cultured human corneal and RPE cells. Human corneal epithelial cells, Human corneal endothelial cells (HCEC), SV40 transfected human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE) were treated without and with ?-IFN for three days. Following fixation with 70% ethanol in 50 mmol glycin buffer, cells were examined for expression of HLA-DP, DQ, DR by indirect staining with mouse anti-human HLA class II monoclonal antibody. Anti-mouse biotinylated secondary antibody and streptavidin conjugated alkaline phosphatase were used. The binding antibody were visualized by a red precipitate at the antigen site. All cell types showed upregulation of HLA expression after ?-IFN treatment. Magnification 100x.

4.1.2 Expression of CD40

The cultivation of human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE) were performed as the material and methods part. Some cultured cells were supplemented with γ-IFN 1000 U/ml. Three days later, the confluent cells were used for immunoperoxidase staining with mouse anti-human monoclonal antibody. Cultured human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE), were separately analyzed, considering for the expression of CD40. The SV40 transfected HCEC and RPE cells were negative for CD40 expression. Approximately 95% human corneal endothelial cells (HCEC) indicated extremely high positive staining. No doubt, human corneal epithelial cells remained the highest positive results, almost 100%. Furthermore, CD40 antigen expression was determined after γ-IFN treatment, all kinds of the cells indicated upregulation of CD40 expression. After γ-IFN stimulation, CD40 expression was enhanced to probably 5% positive staining on human retinal pigment epithelial cells (RPE) and immortalized human corneal endothelial cells (SV40 transfected HCEC). Particularly, about 100% positive staining were observed on human corneal epithelial cells and human corneal endothelial cells (HCEC). CD40 antigen was detected not only on the cell membrane, but also in the cytoplasm. So the positive cell showed more dense staining than without γ-IFN induction. The results concerning of CD40 expression are summarized in Table 4.2.

	Human corneal epithelial cells	**HCEC**	**SV40 transfected HCEC**	**Human RPE**
- γ-IFN	++++	+	-	-
+γ-IFN	++++	++++	+	+

Table 4.2: Immunohistochemical determination of CD40 expression on cultured human corneal and retinal cells. Cells were treated or not with γ-IFN (γ-interferon) 1000 U/ml; HCEC: human corneal endothelial cells; RPE: retinal pigment epithelial; SV40 transfected HCEC: immortalized human corneal endothelial cells. Staining was rated as - (negative), + (weak, <25%), ++ (intermediate, 25~50%), +++ (strong, 50~75%), ++++ (very strong, 75~100%).

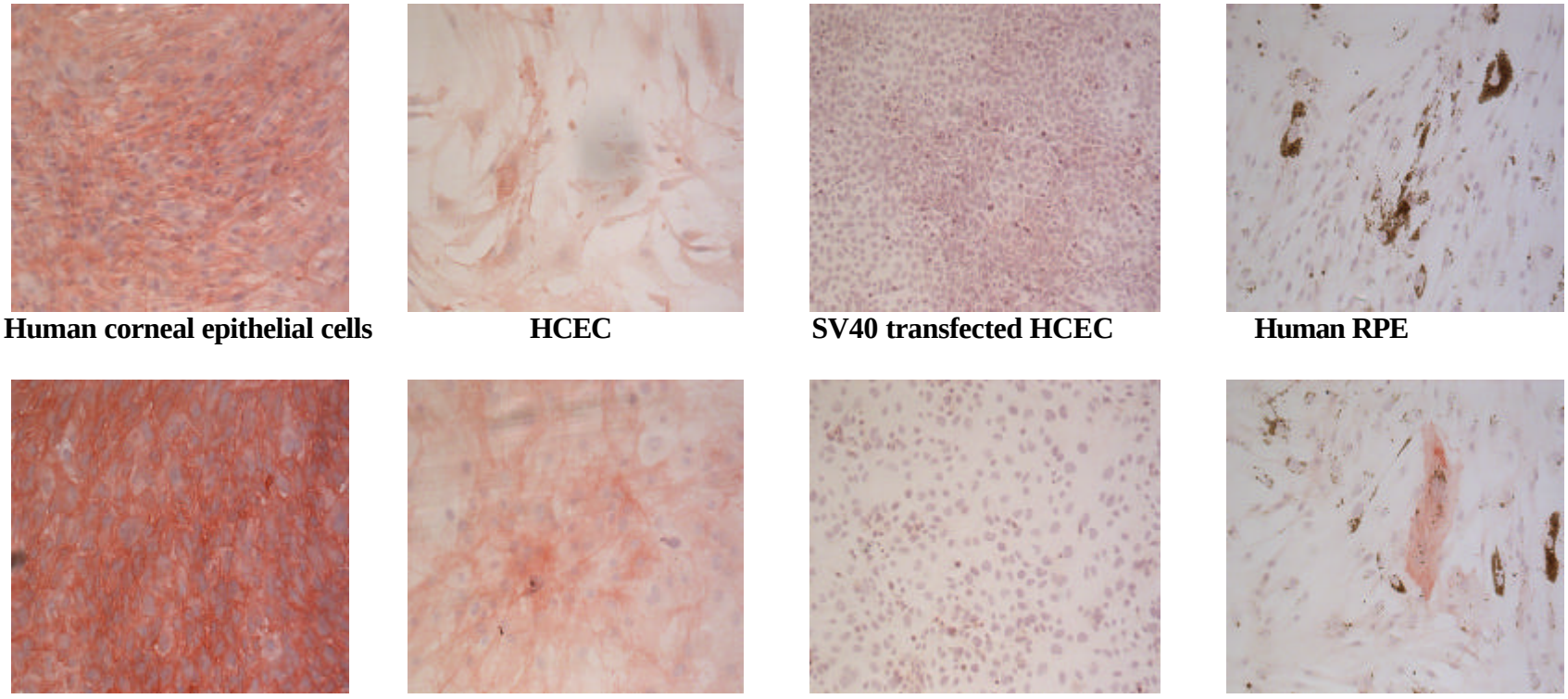

Fig. 4.2: Expression of CD40 molecule on cultured human corneal and RPE cells. Human corneal epithelial cells, human corneal endothelial cells (HCEC), SV40 transfected human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE) were cultivated and stained as described in **Fig. 3.1**. Expression of CD40 occurred in the corneal cell types. In human corneal epithelial and endothelial cells expression was upregulated by ?-IFN treatment, whereas the culture of transfected HCEC no upregulation was seen. Human RPE did not show CD40 expression. Only after ?-IFN treatment, some stained cells appeared within the culture. Magnification 100x.

4.2 Cocultured cells

4.2.1 FACS analysis

Human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE), were cultivated as described in the material and method section. One part of the cells was treated with γ-IFN (1000 U/ml) for three days. After cultures became confluent on the culture cluster bottom, purified fresh PBMCs (peripheral blood mononuclear cells) and anti-CD28 monoclonal antibody (at a concentration of 1µg/ml) were simultaneously provided into all the culture wells, and cultures were continued without γ-IFN treatment. The negative control sample was performed only with PBMCs. As positive control cells, the PBMCs were cocultivated with immobilized anti-CD3 monoclonal antibody (4µg/ml) and anti-CD28 monocloanl antibody (1µg/ml). All the cells were cocultivated with PBMC for two days, then the suspended cells were carefully harvested from the cocultures for FACS analysis (fluorescence activated cell sorter) with anti-$CD3^{FITC}$, anti-$CD69^{PE}$ monoclonal antibodies to quantify the percentage of activated T lymphocytes (Figs. 4.3-4.6) ***LR*** (lower right side) means CD3 positive but CD69 negative T lymphocytes; ***UR*** (upper right side) shows both CD3 and CD69 positive cells, representing activated T lymphocytes (Fig 4.3).

As shown in Table 4.3, after coculture with different cells, the highest level of response was obtained with human corneal epithelia cells pre-stimulated with ?-IFN, which induced CD69 expression (Fig 4. 5). In contrast, human retinal pigment epithelial cells (RPE) seemed to have little or no effect on T lymphocyte activation. It was interesting that, there was no distinct difference of T lymphocyte activation between the cells treated with ?-IFN and without ?-IFN. Human corneal endothelial cells (HCEC) and immortalized human corneal endothelial cells (SV40 transfected HCEC), also could stimulate T lymphocyte to upregulate CD69 on the cell surface, but not as strongly as human corneal epithelial cells.

	both CD3 ^{FITC}, CD69 ^{PE} (percentage of activated T cells)
Human corneal epithelial cells+?-IFN	24%
Human corneal epithelial cells	27%
Human corneal endothelial cells (HCEC) +?-IFN	10%
Human corneal endothelial cells (HCEC)	11%
Immortalized Human corneal endothelial cells (SV40 transfected HCEC) +?-IFN	18%
Immortalized Human corneal endothelial cells (SV40 transfected HCEC)	18%
Human retinal pigment epithelial cells (RPE) +?-IFN	7%
Human retinal pigment epithelial cells (RPE)	5%
Untreated PBMCs (negative control)	1%
PBMCs +a CD3+a CD28 (positive control)	*1: 36%, *2: 71%

Table 4.3 FACS (fluorescence activated cell sorter) analysis. Human corneal epithelial cells, HCEC, SV40 transfected HCEC, RPE were cultivated. One part of the cells was treated with γ-IFN (1000 U/ml) for three days. Fresh purified PBMCs (peripheral blood mononuclear cells) and anti-CD28 monoclonal antibody (1µg/ml) were simultaneously provided into all the culture well, and cultured continued without γ-IFN. The negative control sample contained only PBMCs. As positive control cells, PBMCs were cultivated with immobilized anti-CD3 (4µg/ml) and anti-CD28 (1µg/ml) monocloanl antibodies. After œcultivation for two days, cells in suspension were harvested for FACS analysis with mouse anti-human $CD3^{FITC}$, $CD69^{PE}$ monoclonal antibodies to quantify the percentage of activated T lymphocytes. Percentages of activated T lymphocytes were calculated from the data shown in Figs. 4.3-4.6 and represent the $CD3^{FITC}$ and $CD69^{PE}$ positive population as the percentage of total $CD3^{FITC}$ positive cells.
*1 activate T cells in lymphocyte gate Fig. 4.3, *2 activated T cells in monocyte gate Fig. 4.4

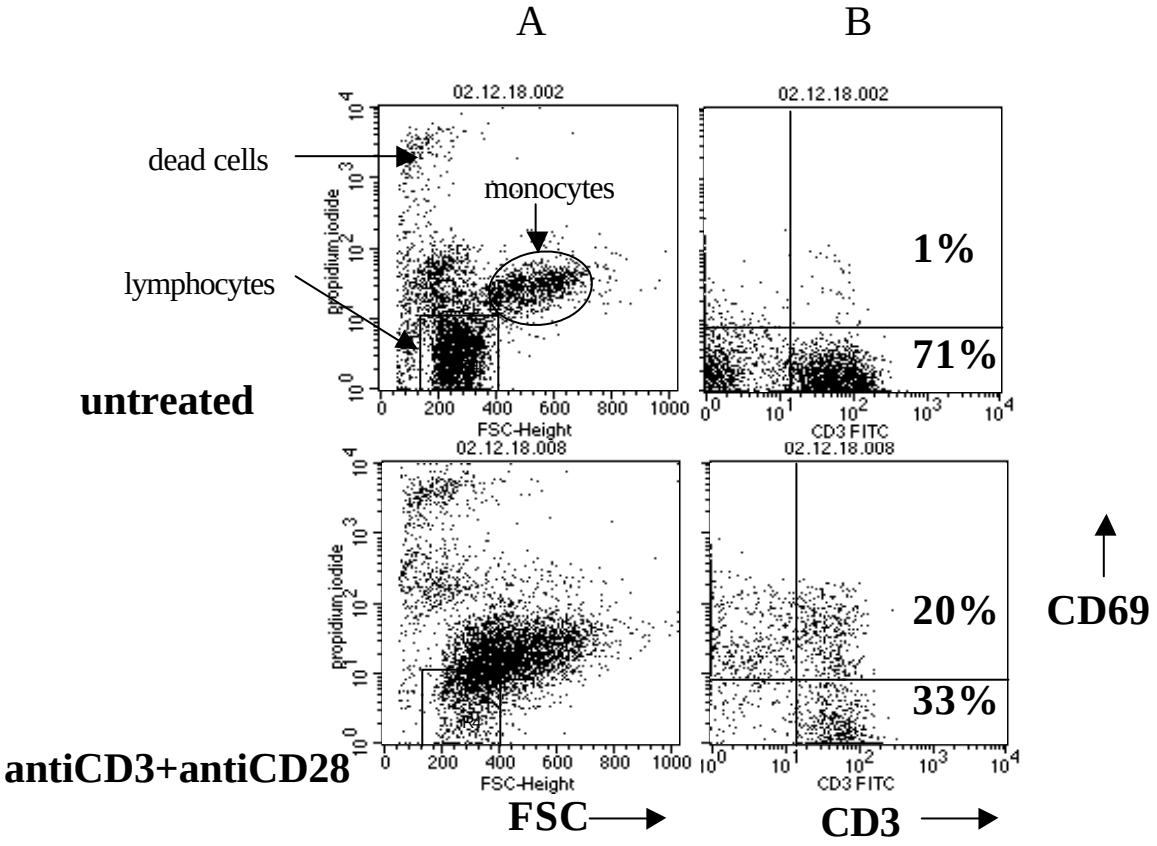

Fig. 4.3. PBMCs gated on lymphocytes. PBMCs were purified by Histopaque 1077 from healthy blood donor. A dot plots comparing forward scatter: FSC (cell size) and Propidium Iodide (PI)*. B dot plots comparing CD3 ^{FITC} and CD69 ^{PE} monoclonal antibodies. PBMCs in untreated were cultured for two days without stimulatory antibodies as negative control. The number in upper right side (percentage of total gated cells) represents both CD3 ^{FITC} and CD69 ^{PE} positive cells (activated T lymphocytes). The number in lower right side represents CD3 ^{FITC} positive and CD69 ^{PE} negative cells (nonactivated T lymphocytes). Cells stimulated with anti CD3 and CD28 monoclonal antibodies were cultured with immobilized anti-CD3 (4 µg/ml) and anti-CD28 (1 µg/ml) for two days as positive control. Soluble cells were harvested and costained for cell surface expression of the T cell receptor (anti-CD3 ^{FITC}) and the early activation antigen (anti-CD69 ^{PE}). Cells were then analyzed for cell size and granularity and fluorescence with an automated flow cytometer (BD FACS-Calibur).
* Box indicates the cells gated for fluorescence analysis.

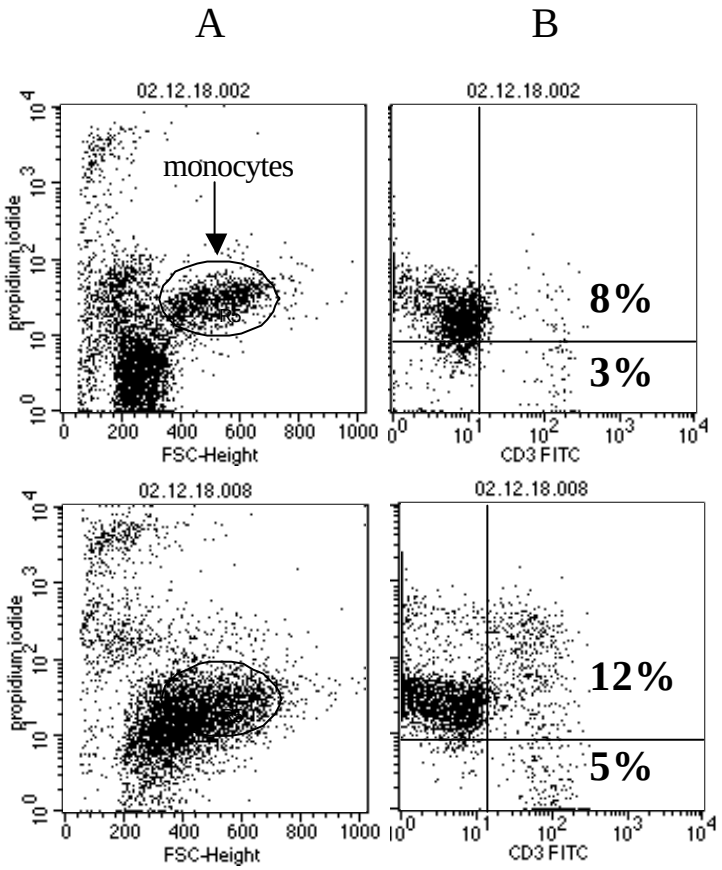

Fig. 4.4. PBMCs gated on monocytes. PBMCs were purified and cultivated as described in Fig. 4.3.

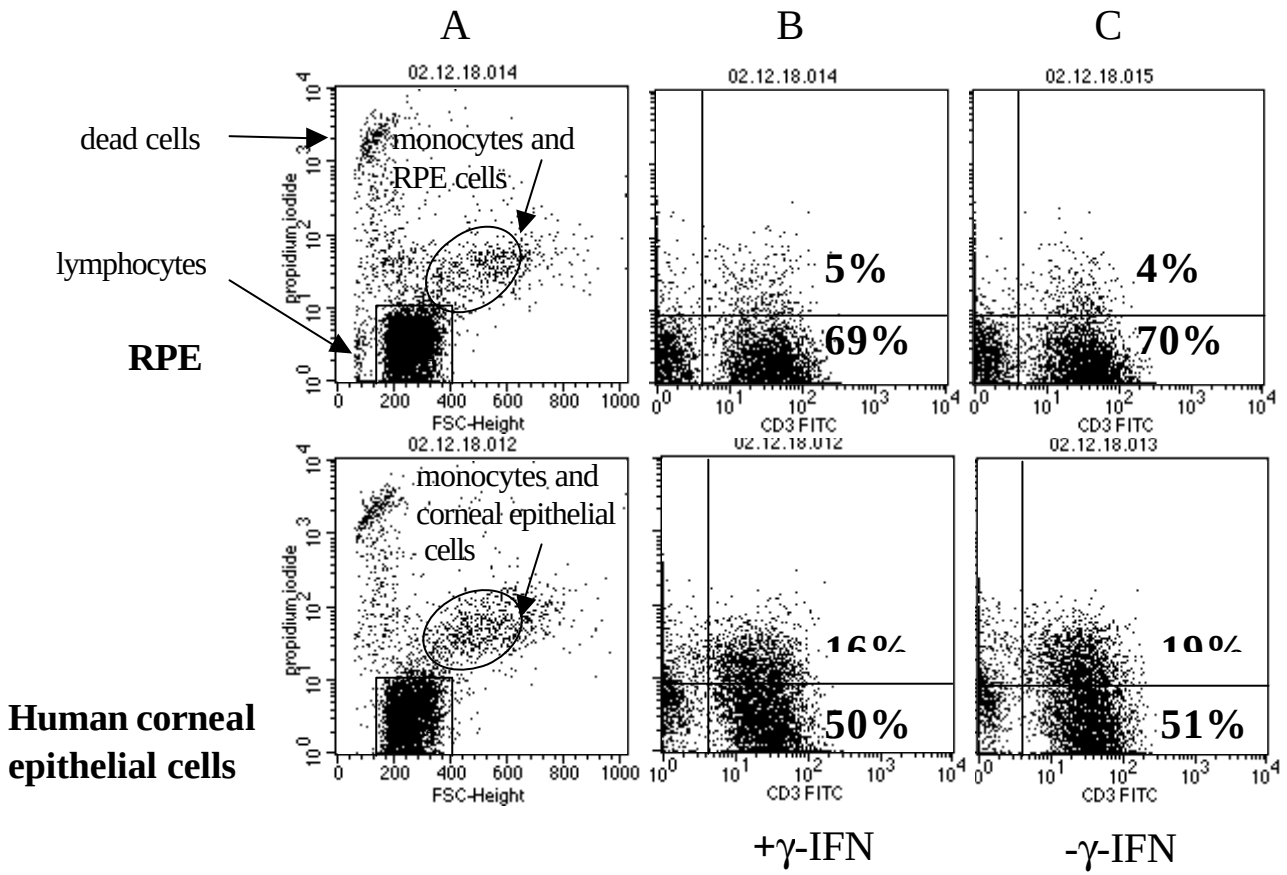

Fig. 4.5. PBMCs gated on lymphocytes cocultivated with RPE (retinal pigment epithelial cells) and human corneal epithelial cells. PBMCs were purified as before. RPE and human corneal epithelial cells were pretreated with γ-IFN (B) and without γ-IFN (C) for three days. Fresh purified PBMCs were cocultivated with RPE and human corneal epithelial cells. Two days later, Soluble cells were harvested and costained for cell surface expression of the T cell receptor (anti-CD3 ^{FITC}) and the early activation antigen (anti-CD69 ^{PE}). Cells were then analyzed for cell size and granularity and fluorescence with an automated flow cytometer (BD FACS-Calibur). The number in upper right side (percentage of total gated cells) represents both CD3 ^{FITC} and CD69 ^{PE} positive cells (activated T lymphocytes). The number in lower right side represents CD3 ^{FITC} positive and CD69 ^{PE} negative cells (nonactivated T lymphocytes).

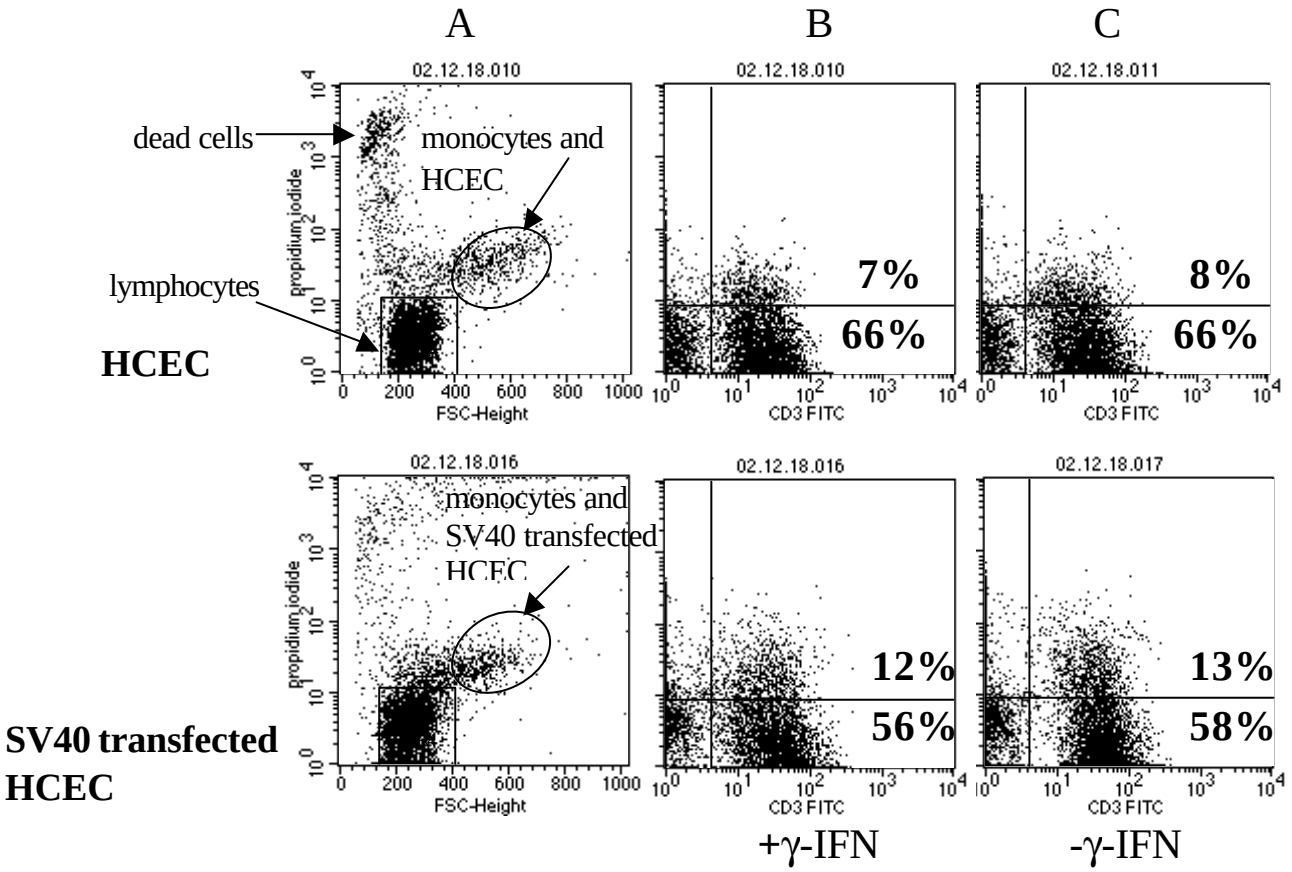

Fig. 4.6. PBMCs gated on lymphocytes cocultivated with human corneal endothelial cells (HCEC) and SV40 transfected human corneal endothelial cells (SV40 transfected HCEC). Cells were cocultured and analysized as Fig. 4.5.

4.2.2 HLA-DP, DQ, DR expression

Cultured human corneal epithelial cells, human retinal pigment epithelial cells (RPE), human corneal endothelial cells (HCEC) and immortalized human corneal endothelial cells (SV40 transfected HCEC) were presented as described in the material and method section. Portion of the cells were induced with γ-IFN 1000 U/ml for three days. All kinds of the cells attached and became confluent on the culture well bottom; purified fresh PBMCs (peripheral blood mononuclear cells) and anti-CD28 monoclonal antibody at a concentration of 1μg/ml were simultaneously supplied into all the culture wells both of γ-IFN treatment and without treatment. All the cells cocultivated with PBMC for two days. Then the cocultivated cell suspension was harvested for FACS analysis (fluorescence activated cell sorter). The cells remained on the culture dish including cultured human corneal epithelial cells, human retinal pigment epithelial cells (RPE), human corneal endothelial cells (HCEC) and immortalized human corneal endothelial cells (SV40 transfected HCEC) and PBMCs were fixed by 70% glycin ethanol. Performed for indirect immunohistochemical staining by mouse anti-human HLA-DP, DQ, DR monoclonal antibody. The HLA class II antigen expression on human retinal pigment epithelial cells (RPE), human corneal epithelial cells, human corneal endothelial cells (HCEC) and immortalized human corneal endothelial cells (SV40 transfected HCEC) cocultivated with PBMC were determined as qualitative analysis. Not only 4 kinds of confluent cells expressed HLA-DP, DQ, DR antigen induced by γ-IFN, was consistent with the results shown before, moreover, the lymphocytes attached on the confluent cell surface have been activated, showed HLA-DP, DQ, DR positive. From the size aspect, lymphocytes were more enlarged than unstained lymphocytes. Furthermore, about the lymphocytes amounts including attached on the confluent cell surface and positive expression of HLA-DP, DQ, DR, the human epithelial cell induced with γ-IFN still indicated the best capability for lymphocytes activation. Then the human retinal pigment epithelial cells (RPE) and human corneal endothelial cells (HCEC). Fewer HLA positive staining cells were observed on immortalized human corneal endothelial cells (SV40 transfected HCEC). It seemed like there was a little enhanced HLA-DP, DQ, DR expression on 4 kinds of cells with γ-IFN induction than without γ-IFN. HLA- DP, DQ, DR antigen was also expressed on the B cells and most monocytes in peripheral blood; so the PBMCs

purified from the same donor were examined. The smear slides were handled as control sample. Nevertheless, there were only some HLA-DP, DQ, DR positive cells on the PBMCs smear slides. The staining effect was not very strong, and the positive cell number was compared with 4 kinds cocultivated cells. (Table 4.4 and Fig. 4.7)

	Epithelial cells	**HCEC**	**SV40 transfected HCEC**	**RPE**
- γ-IFN	Epi +++ PBMC +++	HCEC - PBMC +++	SV40 transfected HCEC - PBMC +	RPE + PBMC+++
+γ-IFN	Epi ++++ PBMC ++++	HCEC ++++ PBMC +++	SV40 transfected HCEC - PBMC +	RPE ++++ PBMC +++

Table 4.4: **Expression of HLA-DP, DQ, DR on corneal cells or retinal cells cocultivated with PBMCs (peripheral blood mononuclear cells).** Human corneal cells and retinal cells were pre-stimulated or not with γ-IFN (γ-interferon) 1000 U/ml for three days. Freshly purified PBMCs and anti-CD28 monoclonal antibody at a concentration of 1μg/ml were cocultivated with all four cell types. After two days, soluble cells were collected for FACS analysis. Attaching cells were fixed with 70% ethanol in 50 mmol glycin buffer. Cells determined for expression of HLA-DP, DQ, DR by indirect staining with mouse anti-human HLA-DP, DQ, DR monoclonal antibody.HCEC : human corneal endothelial cells; RPE: retinal pigment epithelial; SV40 transfected HCEC: immortalized human corneal endothelial cells. Staining was rated as no staining: - (negative), + (weak, <25%), ++ (intermediate, 25~50%), +++ (strong, 50~75%), ++++ (very strong, 75~100%).

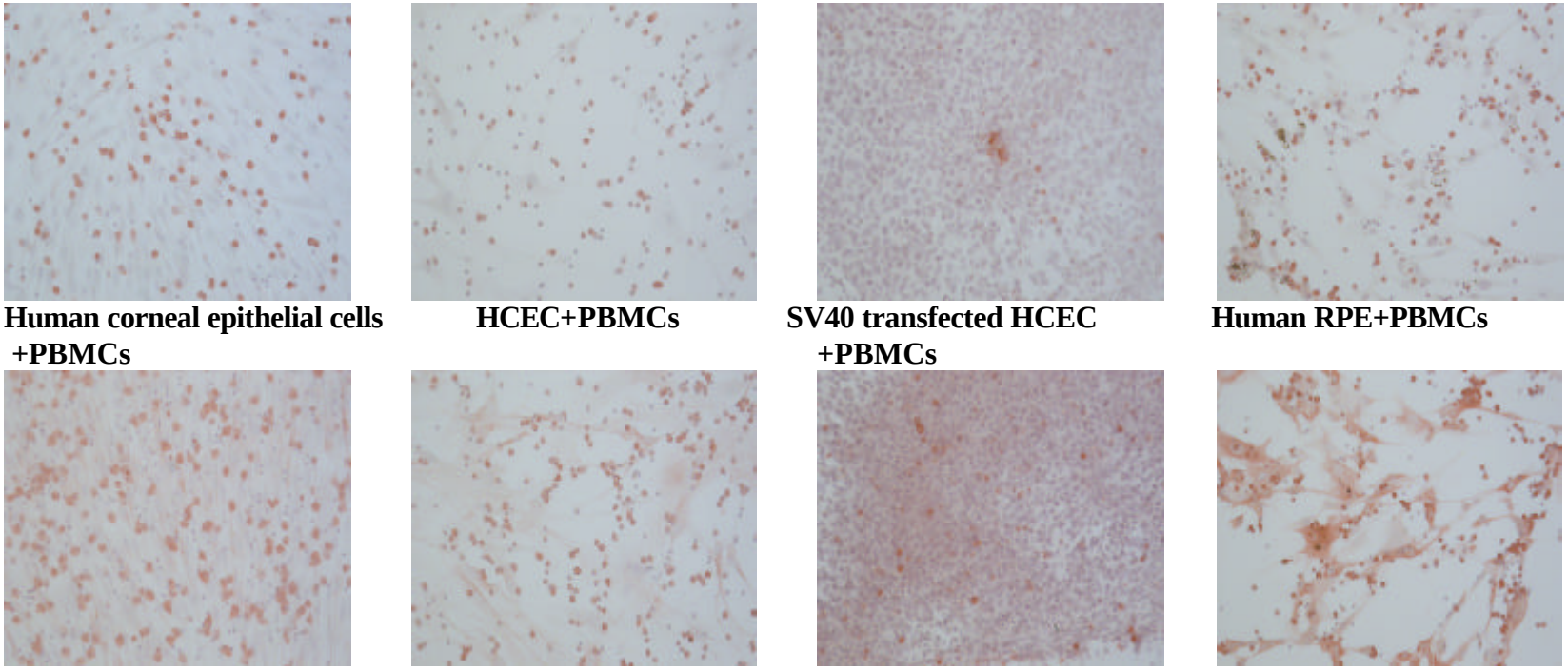

Fig. 4.7: **Expression of HLA class II on human corneal and RPE cells cocultured with PBMCs**. Human corneal epithelial cells, human corneal endothelial cells (HCEC), SV40 transfected human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE) were pretreated or not with ?-IFN for three days. Freshly purified PBMCs were cocultivated with all four types cell. After two days, soluble cells were collected for FACS analysis. Attaching cells were fixed with 70% ethanol in 50 mmol glycin buffer. Cells were determined for expression of HLA-DP, DQ, DR. Primary mouse anti-human HLA class II monoclonal antibody, anti-mouse biotinylated secondary antibody and streptavidin conjugated alkaline phosphatase were used in the indirect staining procedure. The binding antibody was visualized as red precipitate at the antigen site after addition of substrate-chromogen solution. Cells were counterstained with hematoxylin. ?-IFN induced upregulation of HLA expression on all kinds of the cell, including activated T cells. The activated T cells are smaller than the adherent growing corneal and retinal cells. They are seen as small round, red colored dots in these pictures. Magnification 100x.

4.2.3 CD80, 86, 154 expression

The procedure of cell preparation was the same as cocultured corneal cells and retinal cells with PBMCs. Meanwhile, after the cells utilized for FACS analysis were transferred; The remaining cells on the culture well bottom including cultured human corneal epithelial cells, human corneal endothelial cells (HCEC), immortalized human corneal endothelial cells (SV40 transfected HCEC), human retinal pigment epithelial cells (RPE), and PBMCs were fixed by 10% formalin. Then the indirect immunohistochemical staining were respectively accomplished with mouse anti-human CD80, CD86, CD154 monoclonal antibody. None of the cells exhibited CD154 expression, even after induction by γ-IFN. The CD80, CD86 antigens were not expressed on RPE cells with and without γ-IFN. And also no positive lymphocytes were observed. Concerning human corneal epithelial cells, both CD80 and CD86 were expressed very strongly, containing also parts of positive stained lymphocytes. Interestingly, probably not more than 50% CD80 positive cells were presented in cultures of HCEC and less than 5% positive on lymphocytes. But about 5% CD86 positive staining only could be seen on HCEC pre-stimulated with γ-IFN. The HCEC without γ-IFN stimulation and lymphocytes were all negative for CD86. Except for about 95% CD80 positive expression and nearly 50% positive lymphocytes were indicated on SV40 transfected HCEC with and without γ-IFN treatment, no CD86 positive staining was shown. (Table 4.5 and Fig. 4.8-10)

Epithelial cells

	Negative	***CD80***	***CD86***	***CD154***
- γ-IFN	_	Epi +++ PBMC++	Epi ++ PBMC++	Epi - PBMC-
+γ-IFN	_	Epi +++ PBMC++	Epi ++ PBMC++	Epi - PBMC-

HCEC

	Negative	***CD80***	***CD86***	***CD154***
- γ-IFN	_	HCEC ++ PBMC +	_	_
+γ-IFN	_	HCEC + PBMC ++	Epi faint + PBMC -	_

SV40 transfected HCEC

	Negative	***CD80***	***CD86***	***CD154***
- γ-IFN	_	SV40 transfected HCEC++ PBMC++	_	_
+γ-IFN	_	SV40 transfected HCEC++++ PBMC++++	_	_

RPE

	Negative	***CD80***	***CD86***	***CD154***
- γ-IFN	-	-	-	-
+γ-IFN	-	-	-	-

Table 4.5: Immunohistochemical staining of CD80, CD86, CD154 expression on cocultured human corneal and RPE cells. Human corneal cells and retinal cells were pre-stimulated or not with γ-IFN (γ-interferon) 1000 U/ml for three days. The cells were cocultivated with PBMCs (peripheral blood mononuclear cells) and anti-CD28 monoclonal antibody at a concentration of 1μg/ml for another two days. After fixation with 10% formalin. Cells were determined for CD80, CD86, CD154 molecule by indirect staining with mouse anti-human CD80, CD86, CD154 monoclonal antibody. HCEC: human corneal endothelial cells; RPE: retinal pigment epithelial; SV40 transfected HCEC: immortalized human corneal endothelial cells. Staining was rated as no staining: - (negative), + (weak, <25%), ++ (intermediate, 25~50%), +++ (strong, 50~75%), ++++ (very strong, 75~100%).

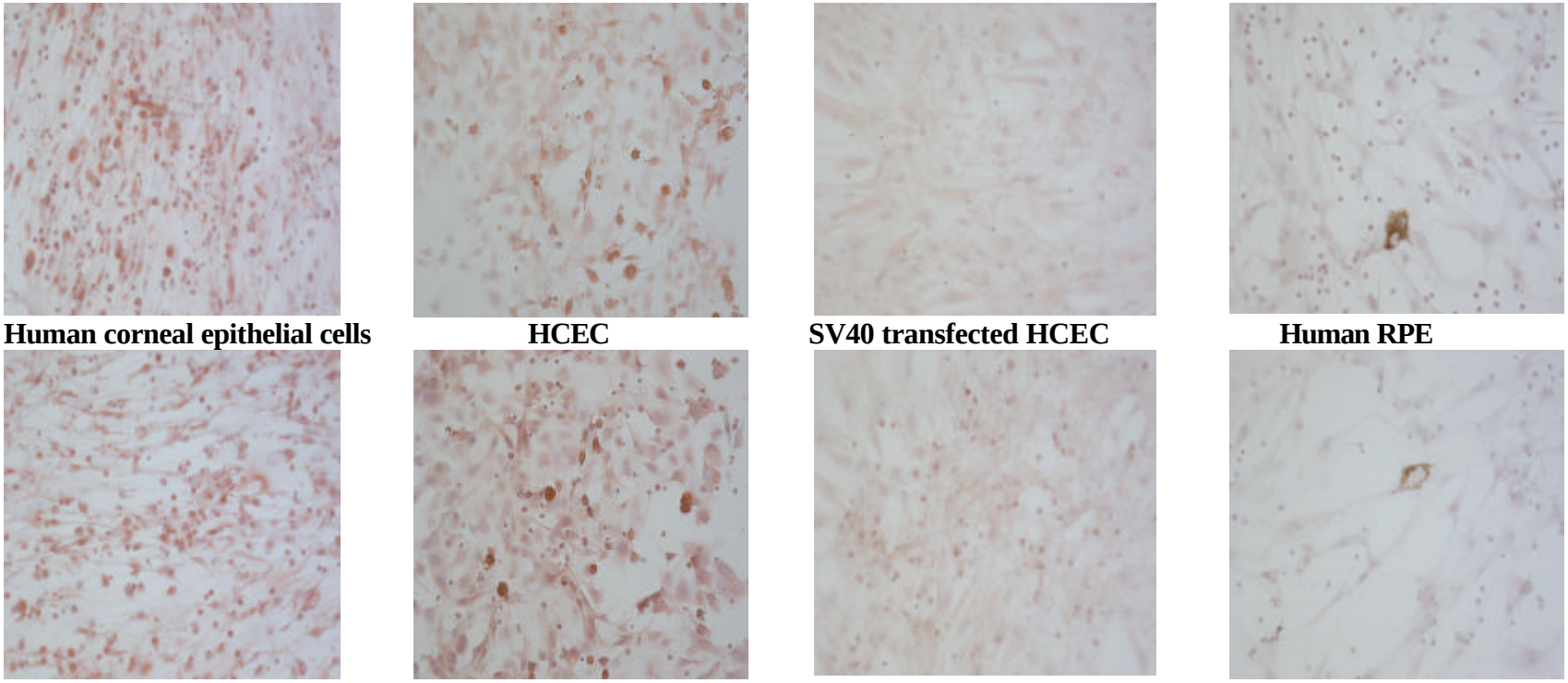

Fig. 4.8: **CD80 expression on human corneal and RPE cells cocultured with PBMCs**. Human corneal epithelial cells, human corneal endothelial cells (HCEC), SV40 transfected human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE) were respectively stimulated or not with ?-IFN for three days. The cells were cocultivated with PBMCs for another two days. After fixation with 10% formalin, cells were analyzed for CD80 expression by indirect staining with mouse anti-human CD80 monoclonal antibody. The staining prodedure was performed as Fig. 4.1. Human corneal epithelial cells, HCEC, SV40 transfected HCEC and cocultured PBMCs were found to be positive. There was no distinct difference between cells with or without ?-IFN treatment. RPE were negative. Magnification 100x.

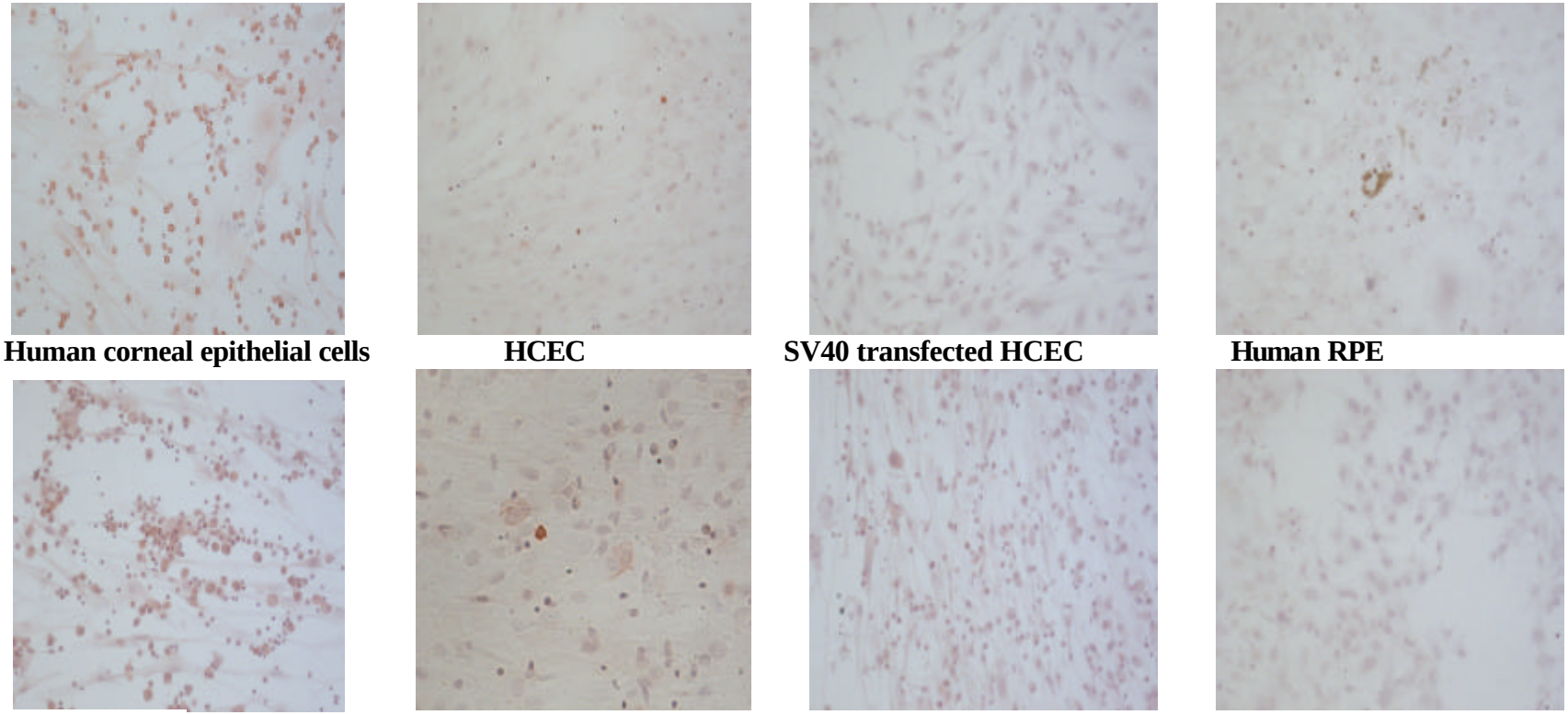

Fig. 4.9: **CD86 expression on human corneal and RPE cells cocultured with PBMCs**. Human corneal epithelial cells, human corneal endothelial cells (HCEC), SV40 transfected human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE) were cocultivated and stained as in Fig. 4.8. CD86 molecule was detected with mouse anti-human CD86 monoclonal antibody. Human corneal epithelial cells with and without ?-IFN treatment were found to be positive. HCEC pre-stimulated with ?-IFN were seen faint staining. The other cells did not exhibit any staining. Magnification 100x.

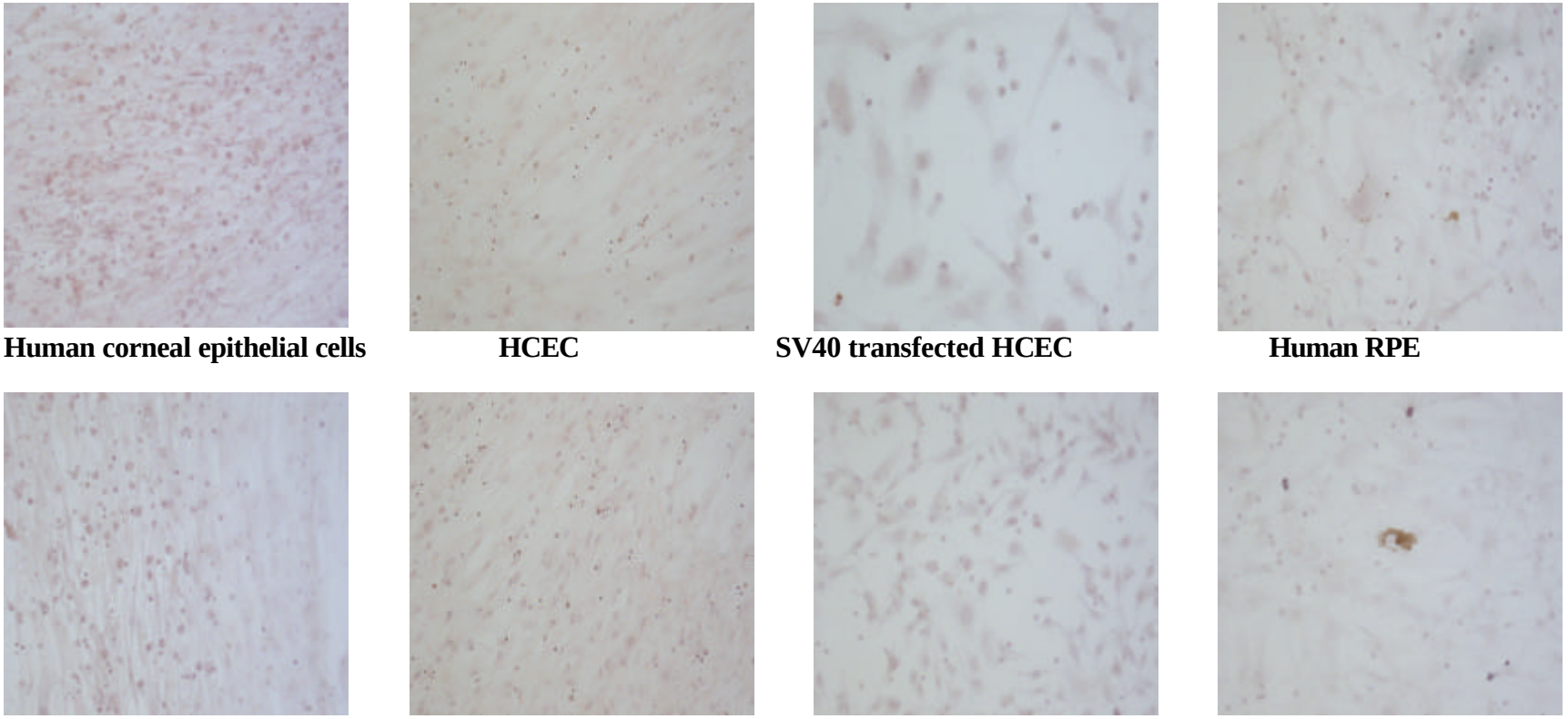

Fig. 4.10: **CD154 expression on human corneal and RPE cells cocultured with PBMCs**. Human corneal epithelial cells, human corneal endothelial cells (HCEC), SV40 transfected human corneal endothelial cells (SV40 transfected HCEC) and human retinal pigment epithelial cells (RPE) were cocultivated and stained as in Fig. 4.8. CD154 molecule was analyzed with mouse anti-human CD154 monoclonal antibody. All the cells were negative for CD154 expression. Magnification 100x.

4.2.4 CD80, 86, 154 expression on corneal epithelial cells

The method of cultivated human corneal epithelial cells were recorded as described in the material and method part. Half of the cells were treated with γ-IFN 1000 U/ml for three days, another part without any treatment. Then all of the cells directly fixed by 10% formalin, following immunohistochemical staining of CD80, CD86, CD154 antigen expression. More than 95% CD80 positive cells were presented in cultures of human corneal epithelial cells either with or without γ-IFN induction. There was no CD86 expression on the human corneal epithelial cells. Compared with CD154 negative expression without γ-IFN treatment, approximately 95% corneal epithelial cells with γ-IFN stimulation showed faint positive staining (Table 4.5 and Fig. 4.11). To investigate further expression of human corneal epithelial cells, PBMCs were respectively cocultivated with human corneal epithelial cells without any treatment for two days, four days, six days. Then the immunohistochemical staining of CD80, CD86, CD154 expression were determined by the attached human corneal epithelial cells. The cocultivated cell suspension was also harvested and smear slides were performed. The same antigen measurement of lymphocytes. It was obviously that from the second day of coculture, human corneal epithelial cells and lymphocytes became strong positive for CD80 expression. Following coculture process, human corneal epithelial cells and lymphocytes increased CD80 expression until the sixth day. From smear slides aspect, whether the lymphocytes number or the positive quantity, there was the best results on the sixth day. Since the second day, CD86 antigen expression exhibited on the human corneal epithelial cells and lymphocytes (more than 50%), but there was no distinct difference following the change of culture period about antigen expression. It was the same phenomena of CD86 positive staining with smear slides. Concerning CD154, there was no faint positive staining until the sixth day. All the results were summarized on the Table 4.6, Fig. 4.12.

	Negative	*CD80*	*CD86*	*CD154*
- γ-IFN	–	+++	–	–
+γ-IFN	–	+++	–	Faint +

Table 4.6: **Immunohistochemical detection of CD80, CD86, CD154 expression on human corneal epithelial cells without** g**-IFN (**g**-interferon). Cultured human corneal epithelial cells were directly fixed with 10% formalin.** Immunohistochemical detection of CD80, CD86, CD154 was performed by indirect staining with mouse anti-human CD80, CD86, CD154 monoclonal antibody. HCEC: human corneal endothelial cells; RPE: retinal pigment epithelial; SV40 transfected HCEC: immortalized human corneal endothelial cells. Staining was rated as no staining: - (negative), + (weak, <25%), ++ (intermediate, 25~50%), +++ (strong, 50~75%), ++++ (very strong, 75~100%).

	2 days	4 days	6 days
CD80	epi +++; PBMC+++	epi ++++; PBMC++++	epi ++++; PBMC++++
CD86	epi ++; PBMC++	epi ++; PBMC++	epi ++; PBMC++
CD154	epi - ; PBMC -	epi - ; PBMC -	epi ++++; PBMC++

Table 4.7: Immunohistochemical detection of CD80, CD86, CD154 expression on human corneal epithelial cells cocultivated with PBMCs (peripheral blood mononuclear cells) respectively for two days, four days and six days. Human corneal epithelial cells were cocultured with freshly purified PBMCs for two days, four days and six days. Immunohistochemical detection of CD80, CD86, CD154 was performed by indirect staining with mouse anti-human CD80, CD86, CD154 monoclonal antibody. HCEC : human corneal endothelial cells; RPE: retinal pigment epithelial; SV40 transfected HCEC: immortalized human corneal endothelial cells. Staining was rated as no staining: - (negative), + (weak, <25%), ++ (intermediate, 25~50%), +++ (strong, 50~75%), ++++ (very strong, 75~100%).

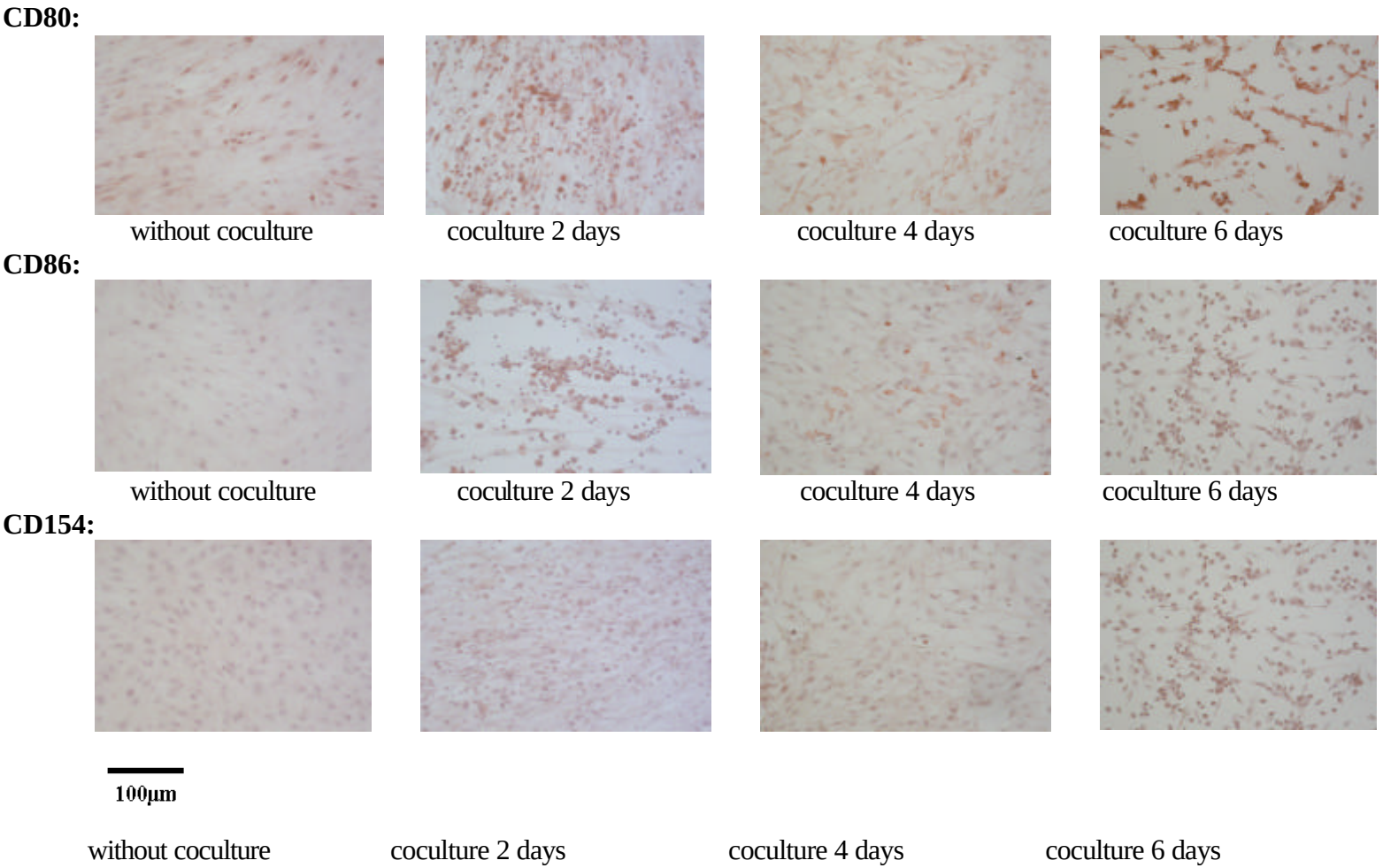

Fig. 4.11: CD80, CD86 and CD154 expression on human corneal epithelial cells and human corneal epithelial cells cocultured with PBMCs. Part of cultured human corneal epithelial cells were directly fixed with 10% formalin. PBMCs were respectively cocultivated with another part of the cells for two days, four days and six days. Immunohistochemical detection of CD80, CD86, CD154 was performed as Fig. 4.1. CD80 was expressed in all cultures. Expression of CD86 and CD154 apeared only after cocultured with PBMCs was seen from day two. Expression of CD154 appeared only after day six. Magnification 100x.

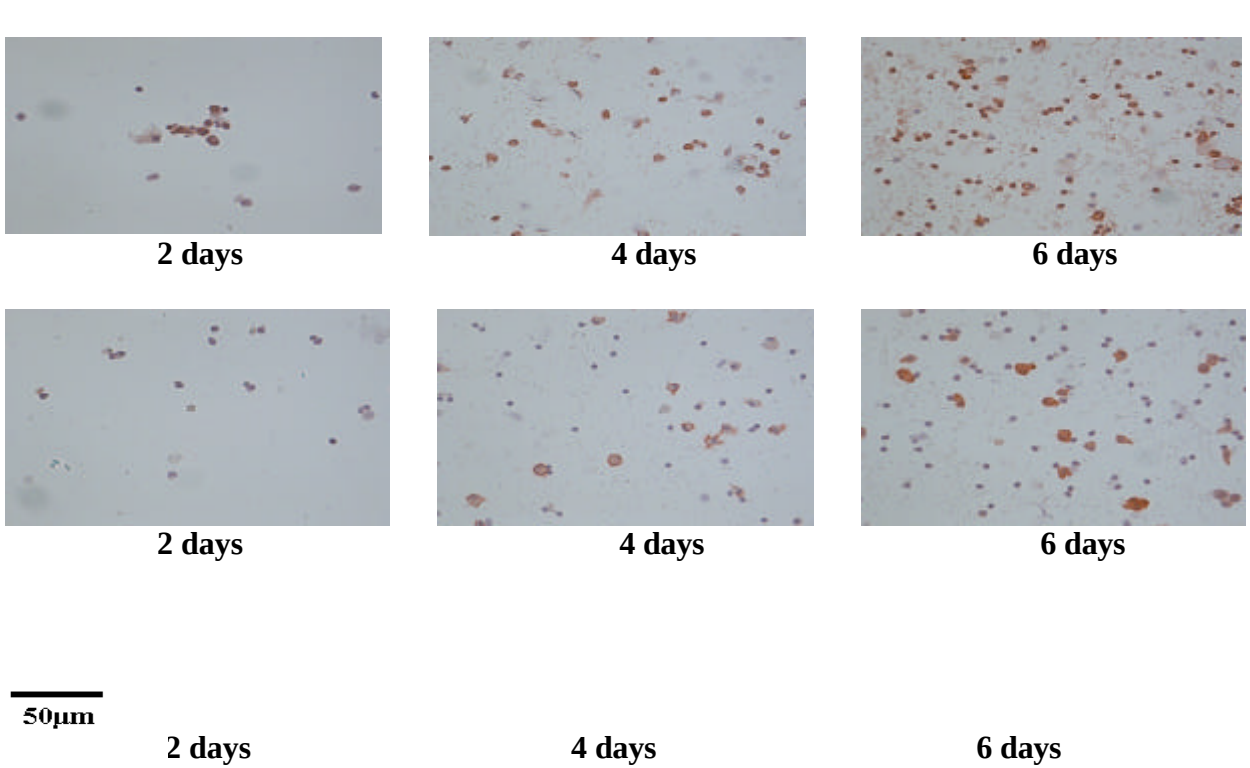

Fig. 4.12: CD80, CD86 and CD154 expression on PBMCs cocultivated with human corneal epithelial cells. PBMCs were respectively cocultivated with human corneal epithelial cells for two days, four days and six days. The cocultivated cells were harvested and smear slides were performed. Cells were fixed and stained as before using antibodies against CD80, CD86, CD154. The cells in the supernatant showed strong positive reaction with CD80 during culture; CD86 was expressed, but no change was observed during culture time; CD154 was not expressed until day six. Magnification 200x.

4.2.5 Corneal transplantation rejection section staining

The corneal transplantation rejection grafts and keratoconus grafts were used for paraffin section. Method in detail was described in material and method part. Then 9 rejection corneas and 5 keratoconus sections were analyzed by immunohistochemical staining using monoclonal antibody for CD4, CD8, CD40, CD154, HLA-DP, DQ, DR. All five control corneal buttons (keratoconus) were negative for the set of antibody markers studied except for CD8 antibody. No inflammatory cells were seen and no HLA class II molecules were detected on the surface of epithelium, stroma or endothelium of keratoconus sections. On the opposite, the rejected, failed grafts showed strong staining for HLA-DP, DQ, DR (Fig. 4.15). The percentage of HLA class II molecules positivity was found particularly on cells in the basal layers of the corneal epithelium and even on endothelial cells. Also in the stroma, the positive cells were sited on the proliferated neovascular endothelial cells, some positive cells were around vessels, the others were diffusely distributed with the stroma. Especially, nearby the graft margin, the conjunctiva tissue were proliferated and were combined with invaded neovascular, HLA-DP, DQ, DR were exhibited strong positive staining. Throughout 9 from patients shown transplantation rejected corneas, there were three cornea sections indicated faint positive staining on the superficial layer of corneal epithelium with anti-CD40 antibody (Fig. 4.16). Regarding to the expression of CD4 on the rejection corneas, there were only two positive results of nine cornea sections (Fig. 4.13). Faintly stained scaled cells were more nearby neovasculars in the stroma. No staining was found including the epithelium and endothelium. Concerning the CD154 expression, no positive results were determined in epithelium, stroma and endothelium (Fig. 4.17). CD8 antigen expression was positive throughout the whole epithelium. It was also positive on the endothelium for all of the control samples. But there was no staining in the stroma. About the rejection corneas, CD8 antigen were also shown on the entire epithelium and endothelium. Not only that, there were proliferated neovasculars mixed in the stroma, CD8 positive cells were also observed around the vessels. CD8 was also showed on keratoconus grafts, the results could not be compared as control samples (Fig. 4.14).

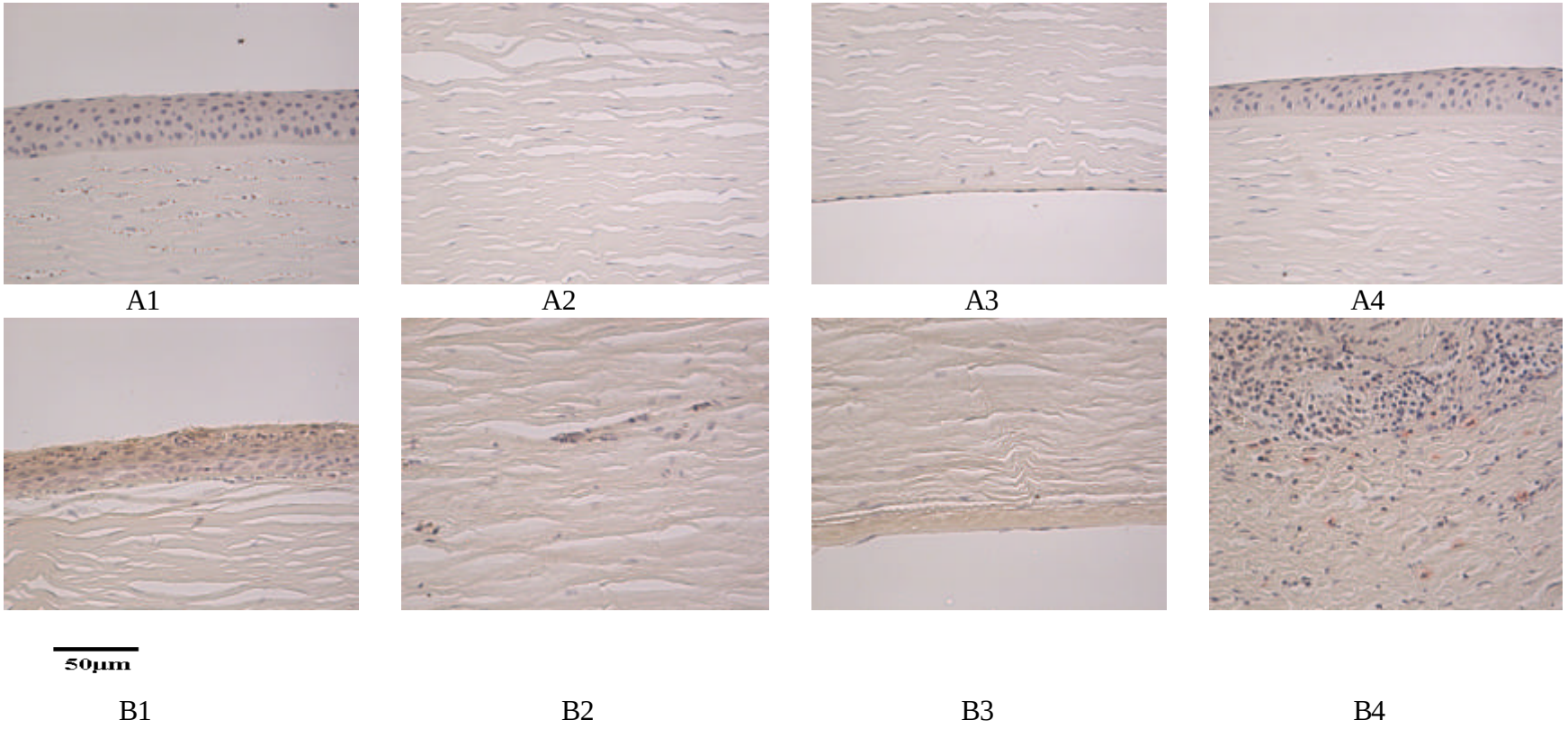

Fig. 4.13: CD4 expression in corneas derived from patients with keratoconus or graft rejection. Slides of paraffin embedded corneas from patients with keratoconus (A1–A4) or graft rejection (B1–B4) were deparaffinized and rehydrated. The specimens were incubated with mouse anti-human CD4 monoclonal antibody. After incubation with biotinylated link antibody and alkaline phosphatase-labelled streptavidin, staining was completed by adding substrate –chromogen solution, resulting in a colored precipitate at the antigen site.
1: epithelial site; 2: stroma; 3: endothelial site; 4: corneal graft edge.
There were no CD4 expressing cells found in corneas derived from patients with keratoconus. In corneas obtained from patients suffering graft rejection, faint CD4 expression was found only near the graft edge (B4). Magnification 200x.

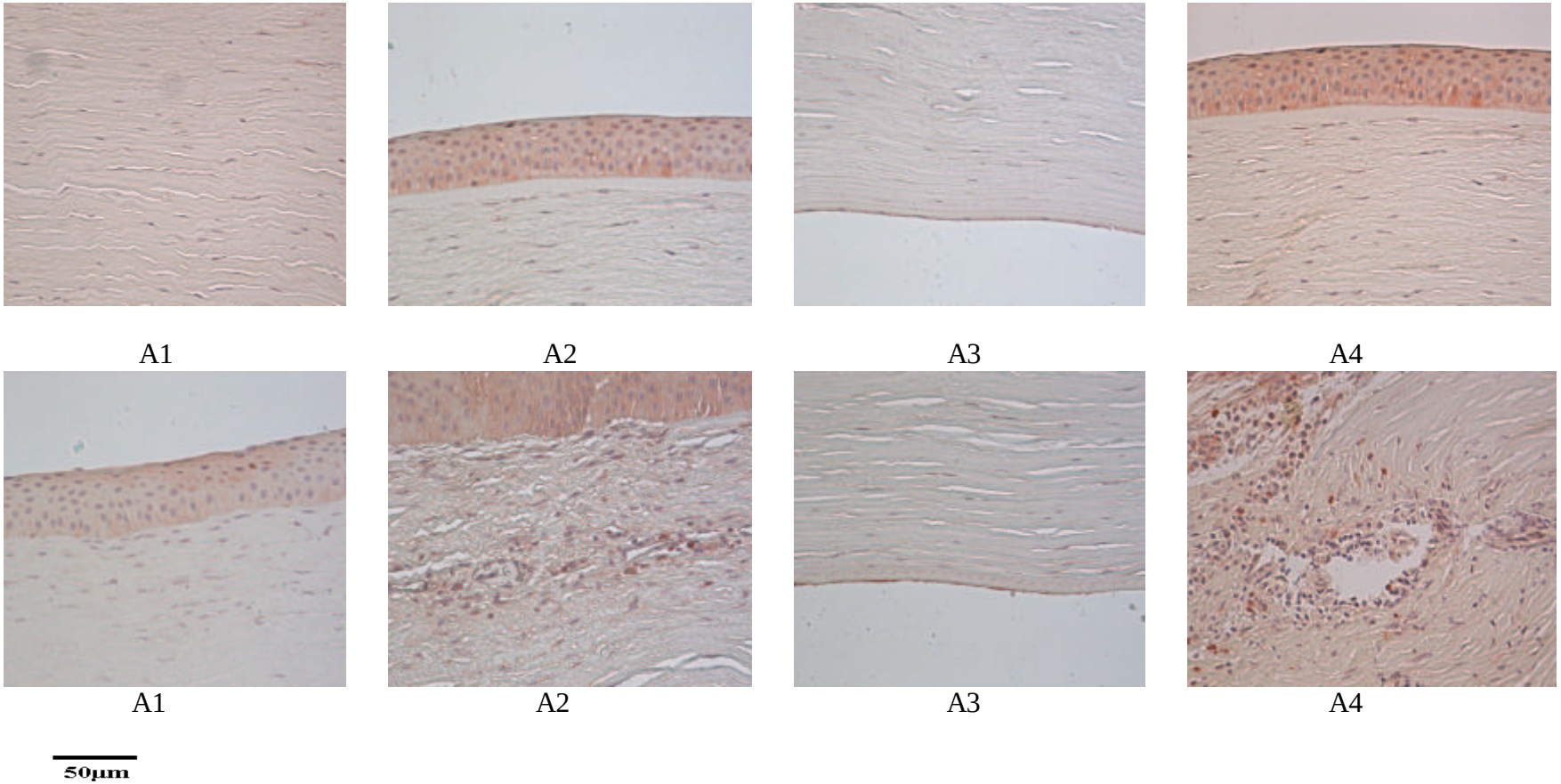

Fig. 4.14: CD8 expression in corneas obtained from patients with keratoconus or graft rejection. Immunohistochemical staining procedure of corneal slides from patients with keratoconus (A1–A4) or graft rejection (B1–B4) were performed as described in Fig. 4.13 using mouse anti-human CD8 monoclonal antibody.
1: epithelial site; 2: stroma; 3: endothelial site; 4: corneal graft edge.
CD8 expression was shown on epithelium (A1), endothelium (A3) and epithelium of graft edge of corneas (A4) from patients with keratoconus. CD8 expressing cells were also found on epithelium (B1), cells around newly formed vessels in the stroma (B2), endothelium (B3). Strong stained cells were also presented on the graft edge (B4) of corneas from patients suffering rejection. Magnification 200x.

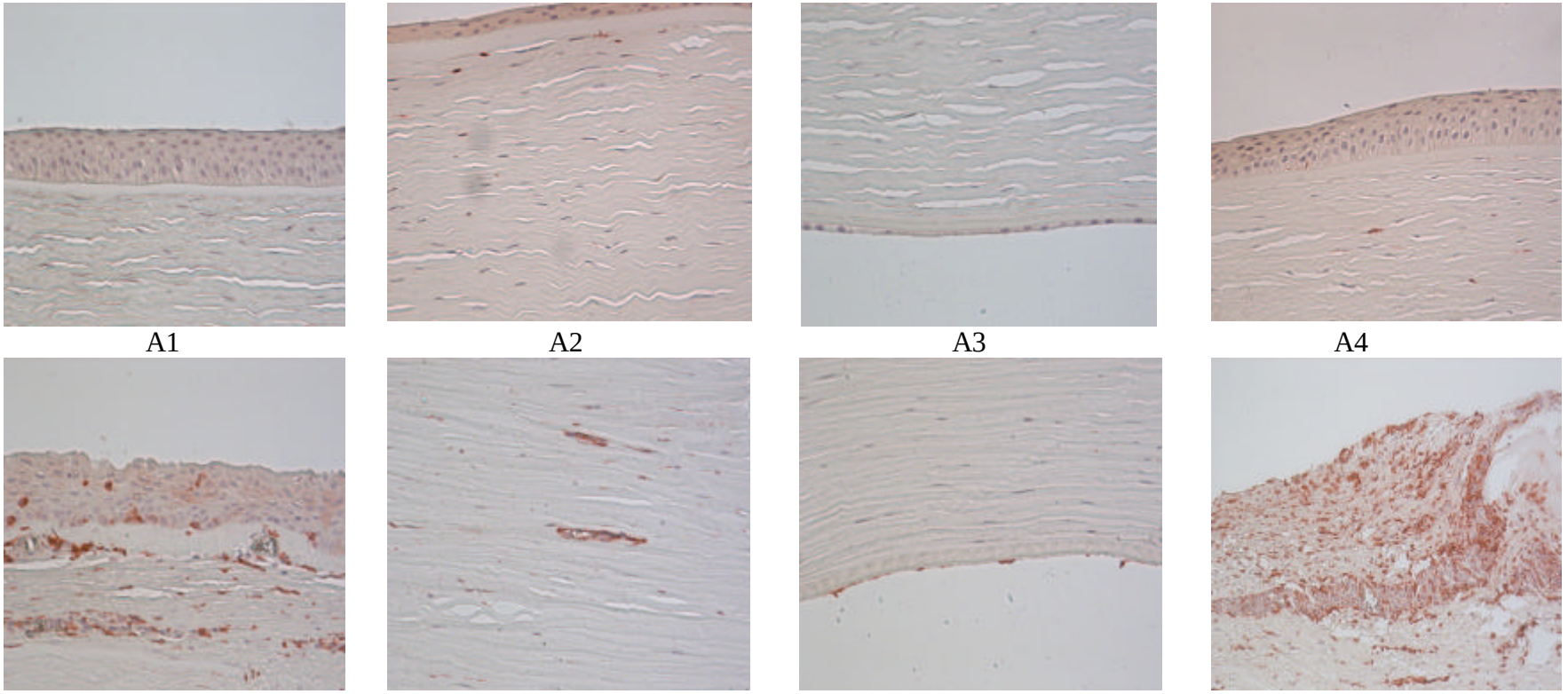

Fig. 4.15: HLA-DP, DQ, DR expression in corneas obtained from patients with keratoconus or graft rejection. Corneas from patients with keratoconus (A1–A4) or graft rejection (B1–B4) were performed immunohistochemical staining procedure as described in Fig. 4.13 using mouse anti-human HLA-DP, DQ, DR monoclonal antibody.

1: epithelial site; 2: stroma; 3: endothelial site; 4: corneal graft edge.

Lessly HLA expression was found on superficial stroma (A2) and stroma of graft edge (A4) of corneas from patients with keratoconus. On the opposite, stronger staining was seen on most of the basal epithelium (B1); diffused staining in stromal (B2); endothelium (B3) and intensive staining on graft edge (B4) of the corneas from patients suffering rejection. Magnification 200x.

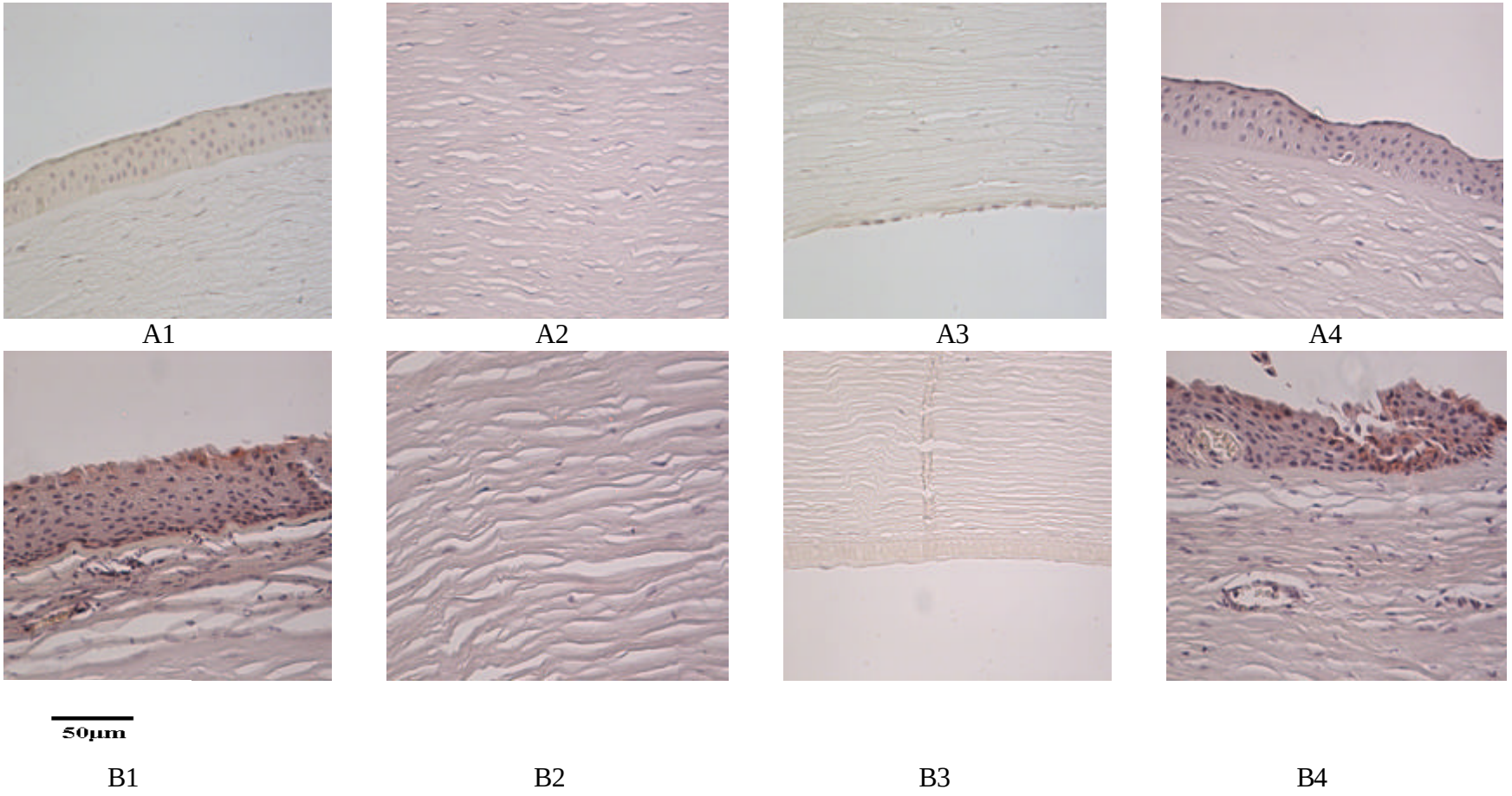

Fig. 4.16: CD40 expression in corneas obtained from patients with keratoconus or graft rejection. Corneas from patients with keratoconus (A1–A4) or graft rejection (B1–B4) were performed immunohistochemical staining procedure as described in Fig. 4.13 using mouse anti-human CD40 monoclonal antibody.
1: epithelial site; 2: stroma; 3: endothelial site; 4: corneal graft edge.
There was no CD40 expression on cells of corneas from patients with keratoconus. Only faint CD40 staining was shown on superficial epithelium (B1) and epithelium nearby graft edge (B4) of the corneas from patients suffering rejection. Magnification 200x.

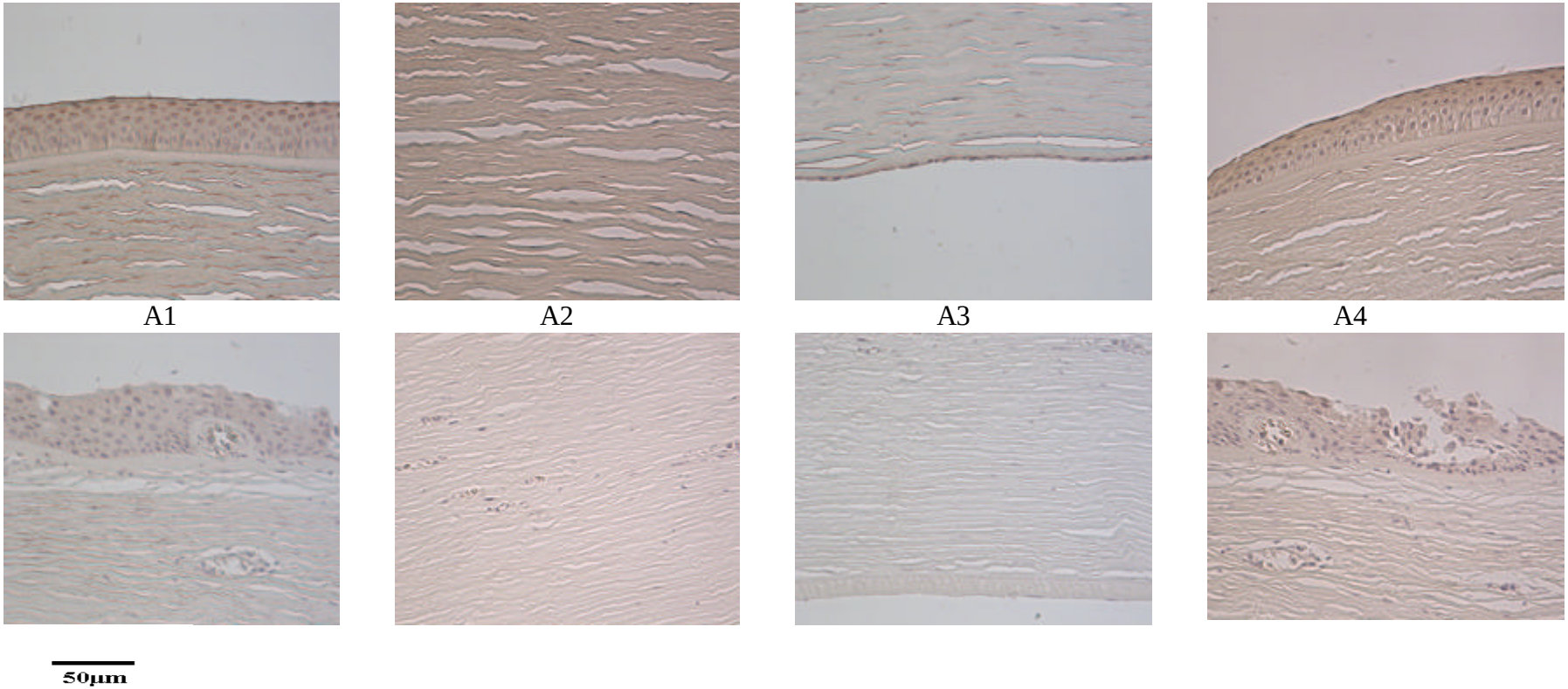

Fig. 4.17: CD154 expression in corneas obtained from patients with keratoconus or graft rejection. Corneas from patients with keratoconus (A1–A4) or graft rejection (B1–B4) were performed immunohistochemical staining procedure as described in Fig. 4.13 using mouse anti-human CD40 monoclonal antibody.
1: epithelial site; 2: stroma; 3: endothelial site; 4: corneal graft edge.
CD154 expression was not seen on cells of corneas from patients with keratoconus and graft rejection. Magnification 200x.

5. Discussion

Keratoplasty represents the most common and successful transplantation of solid tissue in humans. Transplants are usually performed to improve vision and increase the quality of life in patients with damaged or diseased corneas. Corneal transplantation is therefore of significant clinical, biological, and economic relevance. In uncomplicated cases, when the first allografts are performed in avascular beds, the two year survival rate is over 90%. However, approximately 30% of the patients undergoing routine penetrating keratoplasty have a rejection episode, and around 10% of grafts fail because of allograft rejection. Immunological rejection is a leading cause of corneal graft failure. The "high risk" recipient is directly correlated with the severity of corneal vascularisation (Kuffová L et al. 1999). For many reasons, it is likely that the human leukocyte antigen (HLA) alloantigens are involved in corneal graft rejection. They are known to be the main targets for the immunological reactions leading to rejection of transplanted solid organs. It has been concluded that corneal graft rejection was a T lymphocyte mediated immune process (Bertelmann et al. 2002). In this course, T lymphocyte immune responses may be divided into three phases: recognition, activation and effecter mechanisms. T cells are able to recognize foreign peptides in complexes with self-HLA molecules. To activate T cells, two signals are necessary. Signal 1 is delivered through T cell receptor that binds to the allogeneic peptide/HLA complex of the antigen presenting cells (APC), and the CD4 or CD8 coreceptor binds to a constant part of HLA class-II or HLA class-I molecule respectively. Signal 2 or costimulatory molecules include CD28 and its ligands B7-1 (CD80) and B7-2 (CD86), both are expressed primarily on activated antigen-presenting cells. Other T cell surface molecules, such as CD40 ligand (CD154) contribute to signal 2. Following signal 1 and 2, the T cell is fully activated. This study was performed to determine HLA class II expression, and expression of the molecules participating in the process of T cell activation in human corneal epithelial cells, human corneal endothelial cells (HCEC), and immortalized human corneal endothelial cells (SV40 transfected HCEC).

Knowledge of possible reaction ways for immunological processes may be helpful in preventions corneal graft rejection. Furthermore, retinal pigment epithelial cells (RPE) were included in this study, because transplantation of RPE cells, seems to be a promising therapy in cases of RPE degeneration. Until now, no study was performed to

analyze possible RPE rejection processes. Expression of HLA- antigens was found in all four cell types although this expression required ?-IFN (?-interferon) induction in case of human corneal endothelial cells. Therefore, the presence of HLA-antigens on these cells may contribute to T -cell activation. The results concerning this activation will be discussed for each cell type separately.

5.1 Expression of HLA-DP, DQ, DR and T cell activation

5.1.1 Human corneal epithelial cells

About HLA-DP, DQ, DR antigen expression on human corneal epithelial cells, (Iwata et al. 1992) reported there was no class II antigen expressed on human corneal epithelial cells without ?-IFN treatment, but the expression of all these MHC class II antigens were induced on human corneal epithelial cells in culture by ?-IFN. Using the cultivation technique described in this study, HLA-DP, DQ, DR antigens were expressed on human corneal epithelial cells without any stimulation; approximately more than 50% of the cells showed positive staining. Nevertheless, HLA class II expression was still enhanced by ?-IFN treatment, leading to nearly 100% staining on human corneal epithelial cells.

Studies by FACS analysis and immunohistochemical staining demonstrated that human corneal epithelial cells activated human T lymphocytes to express CD69 (Fig. 3.5) and HLA-DR (Fig.3.7). Human corneal epithelial cells induced a higher level of T cell activation than the other cell types, and there was no distinct difference between cells induced or not with ?-IFN. Due to strong HLA classII expression, human corneal epithelial cells can serve as antigen presenting cells, and the T cell receptor can bind to allogeneic peptide/HLA complex. The CD4 coreceptor can bind to a constant part of HLA class II or CD8 can bind to HLA class I molecules respectively. Thereby a first activation signal was transduced into the T cell. The CD28 molecule served as costimulatory signal. CD28 has two known ligands, B7-1 (CD80) and B7-2 (CD86), both of which are expressed primarily on activated antigen-presenting cells. The immunohistochemical staining results showed that CD80 molecules were also expressed on human corneal epithelial cells, so CD28 and B7-1 (CD80)/B7-2 (CD86) could have contributed to signal 2. Following signal 1 and signal 2, the T cells were fully activated.

In some experiments, human corneal epithelial cells without ?-IFN treatment stimulated allogeneic lymphocytes, but always less than ?-IFN treated human corneal

epithelial cells did. In previous study (Iwata et al. 1994), human corneal epithelial cells were regarded as a stimulator, and mixed with responder PBMCs in microtiter plates. Furthermore, one *m*Ci of ^{3}H-thymidine was added to the cultures. 18 hours later, the cultures were harvested on glass fiber filters, ^{3}H-thymidine incorporation was measured by a liquid scintillation counter. This method has the disadvantage that also radioactivy is registered that derived from incorporation into the epithelial cells. Compared with the experimental method, in my study, cultured human corneal epithelial cells attached on the bottom of culture dish. Then the PBMCs cell suspension was supplied into human epithelial cell culture well. After 48 hours, most of the lymphocytes still remained in the cell suspension, few lymphocytes firmly covered on the human epithelial cells. The cell suspension was carefully transferred for FACS analysis. The purpose of my cultivation method was to ensure that only the pure lymphocyte suspension was used to detect the lymphocyte proliferation condition. Because the human epithelial cells still attached on the culture cluster after the lymphocytes were delivered. So the risk that human corneal epithelial cells were mixed into lymphocytes were minimize.

Therefore the method used in this study minimizes the risk of overdetermination of T cell activation but it leads to a certain underestimation. This phenomenon is described in the following. The cocultured PBMCs in the experiment process should be emphasized. The human epithelial cells and PBMCs were cocultivated on the chamber slides. Afterward, the cocultured cells were determined by HLA-DP, DQ, DR monoclonal antibody. The activated lymphocytes could be distinguished by the antibody. The results showed that not only the epithelial cells were positive with HLA class II. Furthermore, correspondingly the lymphocytes attached on the slides became more larger than nonactived lymphocytes, more important is that most of the attaching lymphocytes were positive for HLA class II. Hereby, in case of the most activated lymphocytes were attached on the coculture well, the cell amounts used for FACS analysis were decreased. It means, actually, the effect of lymphocyte activation by human epithelial cells has been underestimated in FACS analyses, due to cell adhesion. In fact, the lymphocytes activation impression was more larger than determined. A larger number of adherent lymphocytes were observed in cocultures with human corneal epithelial cells than in cocultures with other cells (Fig. 4.7), consistent with a strong activation - and proliferation - of lymphocytes by these cells.

5.1.2 Human corneal endothelial cells (HCEC)

In contrast to corneal epithelial cells, cultured cornal endothelial cells exhibited HLA expression only after ?-IFN induction. The result was similar with former results from EL-Asrar et al. (1989). The difference concerns the material and method which had been used by these authors. They excised 3 mm corneal scleral margins, and divided them into small parts. The pieces of cornea were directly placed into culture medium and incubated with recombinant ***Escherichia coli***-derived interferon-gamma in a dose of 5 mg/l and 50 mg/l. Four days later, the corneal pieces were used to prepare frozen section for immunoperoxidase staining of the corneal cell layers. Our laboratory has established HCEC cell cultures, and we have used these cultures to evaluate the profiles of HCEC separated from the original corneal tissue. It could be found that in my study, that investigation of monolayer cell cultures are a simple method which is more directly and more convenient to observe the behaviour of HCEC.

T cell activation in cocultures was determined by FACS analysis. The percentage of activated lymphocytes was lower in HCEC cocultures than in SV40 transfected HCEC and human corneal epithelial cells but higher than in RPE colcultures (Fig. 4.5). This is consistent with the finding that there was no abundant expression of costimulatory molecules, such as CD80, CD86, on HCEC (Figs. 4.8 and 4.9). Evidently, the activation signal was not strongly delivered to lymphocytes.

5.1.3 Immortalized human corneal endothelial cells

HLA class II antigen expression by immortalized human corneal endothelial cells (SV40 transfected HCEC) was analyzed for the first time. It was found that the cells expressed HLA-DP, DQ, DR, only after treatment with ?-IFN. Therefore there was no difference between transfected and nontransfected HCEC concerning HLA-DP, DQ, DR expression. Nevertheless only less than 25% SV40 transfected HCEC exhibited HLA expression. It was appealing that if the cells were in subconfluent condition, the HLA-DP, DQ, DR positive staining was observed distinctly. On the opposite, after the SV40 transfected HCEC ultimately became confluent, the HLA class II positive staining was difficult to be found or only occasionally be detected. We could explain that because confluence might be a turning point from the proliferation stage to the differentiation stage. But it seemed there was no significant difference of HLA-DP, DQ,

DR antigens induction from subconfluent and confluent cultures of human corneal epithelial cells, HCEC and RPE cells.

5.1.4 Human retinal pigment epithelial cells (RPE)

In our laboratory, a method has been established for HLA class II antigen induction with ?-IFN on RPE cells (Valtink et al. 1999). Though HLA class II antigens were never examined by immunohistochemical staining method. Like corneal epithelial cells, RPE cells exhibited strong HLA-DP, DQ, DR antigen induction with ?-IFN. From FACS analysis results, RPE cells showed low ability of T lymphocytes activation. In a former report about RPE cells (Jorgensen et al. 1998), Fas ligand (FasL) expression was detected by flow cytometry and immunohistochemistry staining method. The results indicated that RPE cells expressed FasL and induced TCR-independent apoptosis in activated human T cells through Fas- FasL interaction. Retinal pigment epithelial cells may constitute an immunologic functional barrier against potentially harmful T cells. Few years later, Kaetel et al. (2002) continued this research and found that human retinal pigment cells inhibit the proliferation of activated T cells by a cell contact-dependent mechanism. The inhibition of the T cells´ proliferation correlates with a decreased expression of IL2R-ß and – ? chains. The T cells regain their ability to proliferate and increase their IL2R-ß and – ? chain expression within 24 hour after removal from the coculture. It was concluded that the cultured human RPE cells inhibit the proliferation of activated T cells by a process that does not involve apoptosis. It depended on cell contact but the involved surface molecules were not revealed. The proliferation inhibition correlated with a modulation of the T cells´ expression of IL2R (interleukin-2 receptor), and was reversible. In the above mentioned investigation, the human RPE cells were respectively pre-stimulated with IL-2 (interleukin-2) or PHA (phytahematoagglutinin), and then the RPE cells were cocultivated with lymphocytes. But in our study, there was an important difference to this. That was because we directly used human RPE cells for cocultivation with lymphocytes without any stimulator factor of activation. Nevertheless the obtained results were similar with former research. Human RPE cells could not activate resting T lymphocytes. About the question whether RPE cells can induce lymphocytes´ apoptosis and by which mechanism, more investigations are attempted for clarification of the intracellular pathways through which inhibition of lymphocytes is achieved.

5.1.5 Transplantation rejection cornea section

To investigate HLA-DP, DQ, DR antigens expression in cornea transplants which were removed after graft rejection. Ten rejection cornea grafts were examined by immunoperoxidase staining method. The results demonstrated that HLA class II antigens were expressed in all corneal sections. Extensive HLA class II expression was observed on corneal epithelium, stroma, and even on endothelium. Prominent expression on epithelium was seen in the basal layers. In the stroma, some positive cells diffused, some were distributed around vascular, and some HLA positive cells were expressed on the proliferated neovascular endothelial cells. Especially, it has known that corneal transplantation rejection was frequently happened on neovasclar invaded corneal graft. So nearby the rejection graft margin, strong positive HLA class II antigen expression was seen in the proliferated and thickened conjunctiva tissue. It is well known that Langerhans cells, which expressed HLA class II antigens, prominently existing in limbal and peripheral cornea, can be potent APCs (antigen presenting cells). The FACS analysis results of using cultured human corneal epithelial cells treated with or without ?-IFN support this opinion. Regarding to T lymphocytes activation, there was almost no difference from pre-stimulation with ?-IFN or without ?-IFN. It is possible that ?-IFN is produced locally by the interaction of corneal limbus or peripheral epithelial cells especially Langerhans cells in donor tissue with recipient lymphocytes. Then subsequent production of ?-IFN stimulates HLA class II antigens expression on human corneal epithelial cells, which may initiate corneal graft rejection. On the other hand, HLA-DP, DQ, DR expression on cocultivated corneal cells pre-treated with or without ?-IFN and the lymphocytes attaching on these cells were analyzed, showed that HLA-DP, DQ, DR expression occurred on both cell types. The staining results using cocultivated cells supported the previous outcome of independent corneal cells with HLA class II expression. It seemed there was no or little ?-IFN secretion among human corneal epithelial cells and lymphocytes. It could also be hypothesized that the coculture period of two days was not long enough. The grafts described as chronic graft rejections in clinical observation have been accepted much medicine therapy, therefore, the inflammatory and immunology reactions have been occurred repeatedly during a long time. So it can be also hypothesized that enough ?-IFN secretion requires a longer

cocultivation period between corneal epithelial cells and lymphocytes. This purpose need to be confirmed in the next step study.

5.2 CD40/CD154 pathway

CD40, is a 50-kDa integral membrane glycoprotein. It is found on a variety of antigen-presenting and mesenchymal cells, including B lymphocytes, dendritic cells, macrophages, monocytes, microglia, endothelial cells, and epithelial cells (Grewal and Flavell 1996; Bourcier et al. 2000; Brignole et al. 2000). CD154, also known as CD40 ligand (CD40L), CD40 ligand (CD154), is a 39-kDa type II membrane glycoprotein and member of the tumor necrosis factor (TNF) superfamily. It is preferentially expressed on activated CD4+ cells and mast cells, contribute to signal 2 of T cell activation. Over the past several years, the central role of CD40-CD154 interaction in mediating T-cell–mediated immune responses has been firmly established (Grewal and Flavell 1996). CD154-dependent activation of T cells occurs through signaling of CD40 at the level of antigen-presenting cells (APCs), which enhances requisite costimulatory pathways, including expression of B7.1/B7.2 (CD80/CD86) (Yang and Wilson 1996). Accordingly, it has been shown that blockade of the CD40-CD154 pathway is sufficient to induce tolerance to Th1-mediated contact hypersensitivity (Qian et al. 2001).

In the cornea, it is thought that HLA class II usually is expressed on limbal and peripheral cornea epithelium, and not in the epithelial cells of the central cornea. This is similar to CD40 expression, as demonstrated in the sections. Therefore, there was the possibility that CD40 and HLA class II probably have some similar character, and might be expressed on the same epithelial population. To test this possibility, the same experimental procedure was done with CD40 as HLA class II. But the results showed a little difference with HLA class II. Without ?-IFN treatment, RPE and SV40 transfected HCEC were negative for CD40. About 95% HCEC indicated CD40 positive staining. Human corneal epithelial cells still remained nearly 100% positive for CD40 expression. After ?-IFN induction, RPE and SV40 transfected HCEC were enhanced to almost 5% positive expression, HCEC were achieved almost 100% CD40 positive staining. The human corneal epithelial cells, the quantity of CD40 antigen expression increased. The cells were obtained not only in the cell membrane, but also in the cytoplasm- in conclusion- the positive cells showed more dense staining than without γ-IFN induction. No doubt, human corneal epithelial cells were the most sensitive cells

for CD40 among these cultured cells. Foregoing research in normal cornea, Iwata et al. (2002) concluded that the limbal epithelial cells were predominantly positive for CD40. Positive staining was also found to a lesser extent on the cells in the basal layer of peripheral corneal epithelium. Epithelial cells of the central cornea showed no immunoreactivity for CD40 molecule in the sections. But human corneal epithelial cells and HCEC were all negative for CD154 molecule, CD154 antigens could not be determined on RPE and SV40 transfected HCEC. Only after longer culture periods, expression of CD154 became obvious on human corneal epithelial cells.

Comparing with the conclusion, CD40 expression on the rejection cornea epithelium was determined in our study, but not CD154. The rejection corneal grafts were obtained from the second keratoplasty had taken place, a control normal cornea from a keratoconus patient was obtained. It was impossible to examine the corneal epithelial cells of the limbal and peripheral region, because these structures were not explanted. It could be found that three corneal sections of ten rejected grafts showed faint CD40 positive staining on the superficial layer of corneal epithelium. Especially CD40 positive cells were nearby the graft margin. It has well known that, cells expressing CD40 are mainly hematopoietic cells, antigen presenting cells, such as B cells, monocytes-macrophages, and dendritic cells (Iwata et al. 2002). It is preferential expression of HLA-DP, DQ, DR and CD40 on human corneal epithelial cells that implicate the immunological and the proliferative potential function.

In this study, the human corneal epithelial cells were isolated from limbus area of corneal tissue. As has been demonstrated, proinflammatory cytokines such as ?-IFN induced marked HLA class II and CD40 expression on cultured human corneal epithelial cells. Recently, increasing evidence stressed the critical role of CD40/CD40L (CD154) interactions in the regulation of immune responses, especially mediated by T lymphocytes. The ligation of CD40 on the surface of professional APCs (dendritic cells, B cells, macrophages) by CD40L expressed on activated T cells promotes further upregulation of costimulatory molecules (B7.1, B7.2, CD40) expression on these APCs and high IL-12 secretion, which increase their antigen-presentation and costimulatory capacity (Grewal and Flavell 1996; Schoenberger et al. 1998). The CD40:CD154 pathway, initially described as having a role in B-cell activation, has been recognized as a key pathway for T-cell activation as well (Durie et al. 1994). Stimulation of CD40

provides important signals for antibody production by B cells and strongly induces B7 expression on all antigen-presenting cells. In this manner, the CD40:CD154 system may have an important role in T-cell costimulation. Activation of antigen-presenting cells through CD40 also induces the expression of adhesion molecules and inflammatory cytokines that participate in T cell activation. There is the possibility that the CD40-CD40L interaction in corneal epithelium at the limbus may trigger production of proinflammatory cytokines such as ?-IFN and TNF-α released by human corneal epithelial cells.

5.3 CD28 and CD80/CD86 costimulatory signal

It was also demonstrated in this study that CD80, CD86 molecules could be expressed on human corneal epithelial cells after cocultivated with PBMCs. CD80 antigen was expressed on HCEC and PBMC coculture system. CD80, CD86 could not be determined on RPE and SV40 transfected HCEC. Among these cells, human corneal epithelial cells still were the most sensitive for CD80, CD86 expression.

Two lines of evidence highlight the role of the B7:CD28 pathway of T-cell costimulation in transplant rejection. First, drugs that block the interactions between B7 and CD28 molecules can induce long-lasting nonresponsiveness of T cells to alloantigens in vitro. Second, B7 molecules are induced on vascular endothelium within 24 hours after the transplantation of vascularized organs (Hancock et al. 1996). This means that T cells can receive costimulatory signals throughout the transplanted organ itself. Anti-CD80 and anti-CD86 monoclonal antibodies (mAbs) were administered after orthotopic corneal allograft transplantation (Kagaya et al. 2002). The combined use of anti-CD80 and anti-CD86 mAbs was effective in prolonging corneal allograft survival. Few CD80 (+) or CD86 (+) cells were observed in the cornea treated with anti-CD80/CD86 mAbs. These results demonstrated a significant role of CD80 and CD86 costimulatory molecules in corneal allograft rejection.

Considering the expression of HLA class II, CD40, CD80, CD86 on human corneal epithelial cells and FACS analysis results, it could be concluded that human corneal epithelial cells can participate in corneal immune responses as potential antigen presenting cells, including immunological reaction of corneal graft rejection. It is well known that, in humans, rejection of lamellar corneal grafts are rare compared to rejections of penetrating grafts (Khodaoust and Silverstein 1972). This observation

suggested that human corneal endothelial cells play a key role in alloimmunization of corneal graft recipients. It has also been demonstrated that expression of CD40, CD80 on HCEC, and HLA class II and CD86 molecular could be induced by ?-IFN.

5.4 T lymphocytes

From ten rejection corneal sections, only in two samples $CD4^+$ T cells were found nearby the neovascular and graft margin. In contrast, the epithelium from the keratoconus as well as rejection corneas were all positive using of the anti CD8 antibody. It has been reported that cytotoxic T cells ($CD8^+$) play no essential role in rejection of orthotopic corneal allografts in mice (Yamada et al. 2001). Another research (Boisgerault et al. 2001) concluded that only $CD4^+$ T cells activated via indirect allorecognition have the ability to reject allogeneic corneal grafts. Although alloreactive $CD8^+$ T cells are activated via the direct pathway, they are not fully competent and can not contribute to the rejection unless they receive an additional signal provided by professional APCs in the periphery. Then the effect of $CD8^+$ T cells in corneal transplantation rejection should be attempted continuously. About ?-IFN, anti-human interferon-gamma F(ab´)2 (Fabs) antibodies were administered in patients who experienced corneal transplant rejection as eyedrops by Skurkovich et al. (2002). The results showed that the transplant grafts became fully transparent, eye inflammation disappeared. Visual acuity also increased. It means ?-IFN is significant proinflammatory cytokine in corneal transplantation.

All the phenomenon indicated, human corneal epithelial cells dominantly participate in the corneal transplantation rejection process. Then the corneal transplantation rejection process initiation can be supposed. The physiological activation of T lymphocytes involves at least two signals. Foreign peptides derived from proteins are presented to $CD4^+$ T cells (helper T cells) by HLA-DR, DQ and DP of human corneal epithelial cells. When a TCR binds to the allogeneic peptide/HLA class II complex, and the CD4 (in helper T cells) co-receptors bind to a constant part of HLA class-II, the first activation signal is delivered into the $CD4^+$ T cell. Signal 2 results from the binding of constitutively expressed CD28 molecules on T cells to CD80 or CD86 molecules on human corneal epithelial cells. Other T cell surface molecules, such as CD40 ligand (CD154), CD2, LFA1 and ICAM 1, contribute to signal 2. Following signals 1 and 2, $CD4^+$ T cell is fully activated so that the genes encoding lymphokines

and lymphokine receptors are transcribed and translated. When alloreactive $CD4^+$ T cells are activated on recognition of foreign peptide HLA class-II complexes, they proliferate and, at the same time, secrete a set of lymphokines. The alloreactive T cell response seems to be mainly a Th1 (T helper 1) response, with secretion of IL-2 and interferon gamma (γ-IFN). IL-2 helps to sustain proliferation of $CD4^+$. Help from alloreactive $CD4^+$ Th2 cells is required for activation, proliferation and differentiation of alloreactive HLA-specific B cells into plasma cells that -depending on the HLA disparity between donor and recipient -secrete antibodies specific for donor HLA class-I or class-II molecules. γ-IFN continuously stimulate corneal cells, including epithelial, stroma, endothelial and vascular endothelial cells to express antigen and surface costimulatory molecular, such as CD80, CD86, CD40. These molecules contribute and induce an alloimmune response in the recipient graft.

The current studies have demonstrated the activation process of T lymphocytes and have analyzed constitutively expression of HLA class II antigen, the costimulatory molecular which participate T lymphocyte activation, such as CD80, CD86, CD40 on human corneal epithelial cells, HCEC, SV40 transfected HCEC, RPE cultured cells. Furthermore, the upregualted expression of these cells treated with γ-IFN were also determined. It is concluded that human corneal epithelial cells are important potential antigen presenting cells. The corneal transplantation rejection is initiated by corneal epithelial cell contact with T lymphocytes. The presence of HLA class II can be induced on human corneal endothelial cells during its immune destruction. And this observation implicated that induced HLA class II alloantigen expression on corneal endothelium may be a contributing factor in the rejection of corneal allografts.

6. Summary

The main reason for cornea graft failure after keratoplasty is related to graft rejection. Comparable to the situation after solid organ transplantation, activation of T cells may be involved in this rejection process. The aim of the present study was to investigate the possibility of T cell activation by corneal cells. T cell activation requires two distinct signals from antigen presenting cells.

In a first step the expression of HLA antigens was studied in cultured human corneal endothelial and epithelial cells. Human retinal pigment epithelial cells served as control. This examination revealed that HLA expression occurred in corneal epithelial and also endothelial cells. Therefore these cells may serve as antigen presenting cells. In a second step the human corneal cells were co-cultured with peripheral blood cells. Activation of T lymphocytes purified from peripheral blood cells was analysed by immunohistochemical staining and FACS analysis. The co-culture of corneal cells and peripheral blood cells led to expression of CD69, a marker for activated T cells, in part of the peripheral blood cells population. Furthermore expression of CD80 and CD86 was found on human corneal epithelial cells. Human corneal endothelial cells expressed only CD80 but not CD86. CD80 and CD86 may serve as the second signal for T cell activation. Another molecule that can serve as the required second signal for T cell activation is CD154-a ligand of CD40. This molecule could be detected on human corneal epithelial cells but only after prolonged co-culture time of six days.

Additionally to the cell culture experiments, human corneal grafts were investigated that were exchanged after transplant failure. These investigations revealed CD4 positive T cells participated in the corneal graft rejection. Furthermore, CD40 positive cells were determined in the superficial epithelium.

In summary this study has shown that human corneal epithelial cells seem to participate in the corneal transplantation rejection process, as potential antigen presenting cells. The corneal transplant rejection may be initiated by corneal epithelial cell contact with T lymphocytes. The current studies have demonstrated the activation process of T lymphocytes. The costimulatory molecule which participate in T lymphocyte activation, such as CD80, CD86, CD40, CD154 was also exhibited on human corneal epithelial cells, HCEC. The results were consistent with former report that cytotoxic T cells ($CD8^+$) play no essential role in rejection of orthotopic corneal allografts in mice.

Zusammenfassung

Der Hauptgrund für ein Transplantatversagen nach Keratoplastik ist eine Transplantatabstoßung. In Anlehnung an die Situation nach Transplantation solider Organ könnte die Aktivierung von T-Zellen an diesem Abstoßungsprozess beteiligt sein. Ziel dieser Arbeit war es, die Möglichkeit der T-Zell Aktivierung durch korneale Zellen zu untersuchen. Für die Aktivierung von T-Zellen durch Antigen-Präsentierende-Zellen sind zwei unabhängige Signale notwendig.

Im ersten Teil der Arbeit wurde die Expression von HLA-Antigenen in kultivierten kornealen Endothel- und Epithelzellen untersucht. Humane retinale Pigmentepithelzellen dienten zur Kontrolle. Diese Untersuchungen haben gezeigt, dass sowohl in kornealen Endothel- als auch Epithelzellen HLA-Antigen exprimiert werden. Daher können diese Zellen als Antigen-Präsentierende-Zellen fungieren. Im zweiten Teil der Arbeit wurden die kornealen Zellen mit einer T-Zell enthaltenen Fraktion humaner peripherer Blutzellen cokultiviert. Die Aktivierung der T-Zellen wurde sowohl immunhistochemisch als auch durch FACS-Analyse untersucht. Die Cokultivierung führte zur Expression von CD69, einem Marker für aktivierte T-Zellen. Außerdem wurde Expression von CD80 und CD86 in kornealen Epithelzellen nachgewiesen. Korneale Endothelzellen exprimierten nach Cokultivierung CD80, nicht aber CD86. CD80 und CD86 können als zweites Signal für die Aktivierung von T-Zellen fungieren. Ein weiteres Molekül, das als zweites Signal für die T-Zell Aktivierung dienen kann ist CD154; ein Ligand für CD40. Dieses Molekül konnte in kornealen Epithelzellen nachgewiesen werden, jedoch erst nach einer Cokultivierungszeit von mindestens sechs Tagen.

Zusätzlich zu den Untersuchungen an Zellkulturen wurden humane korneale Transplantate untersucht, die nach Abstoßungsreaktionen ausgetauscht worden waren. Diese Untersuchungen zeigten das Auftreten von CD4 positiven T-Zellen bei Abstoßungsraktionen. Ferner wurden CD40 positive Zellen im kornealen Epithel nachgewiesen.

Zusammenfassend hat diese Untersuchung gezeigt, dass das korneale Epithel am Prozess der Transplantatabstoßung beteiligt zu sein scheint und zwar in Form von Antigen-Präsentierenden-Zellen. Die Transplantatabstoßung könnte demnach durch einen Kontakt von kornealen Epithelzellen und T-Zellen ausgelöst werden.

Moleküle, die als notwendiges zweites Signal für die T-Zell Aktivierung dienen können wie CD80, CD86, CD40 und CD154 werden ebenfalls von kornealen Epithelzellen exprimiert.

7. References

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