Critical contribution of CD80 and CD86 to induction of anterior chamber-associated immune deviation

Rintaro Tsukahara1,2, Masaru Takeuchi1, Hisaya Akiba2, Takeshi Kezuka1, Kazuyoshi Takeda2, Yoshihiko Usui1, Masahiko Usui1, Hideo Yagita2 and Ko Okumura2

1Department of Ophthalmology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
2Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

Keywords: anterior chamber-associated immune deviation (ACAID), co-stimulatory molecules, regulatory T cells

Abstract

Intraocular inoculation of antigens induces anterior chamber-associated immune deviation (ACAID), which is mediated by development of regulatory T cells in response to antigen-presenting cells (APC) pre-conditioned by intraocular transforming growth factor-β (TGF-β). In this study, we examined the involvement of T-cell co-stimulatory molecules in this process. To mimic the intraocular APC, thioglycollate-elicited peritoneal exudate cells (PEC) were pre-treated with TGF-β in vitro. Expression of CD80, CD86, OX40 ligand (OX40L) and CD70 was analyzed by flow cytometry. Contribution of these molecules to co-stimulatory activity of TGF-β-treated PEC on antigen-stimulated T-cell proliferation and cytokine production was determined by inhibition with blocking antibodies in vitro. Contribution of CD80 and CD86 to induction of ACAID was determined by the administration of blocking antibodies at intraocular antigen inoculation in vivo. TGF-β-treated PEC expressed CD80 and CD86 but not OX40L or CD70. Antigen-stimulated T cells proliferated and produced IL-10, but not IFN-γ, in response to co-stimulation by TGF-β-treated PEC, which was abrogated by blocking antibodies against CD80 and CD86. Induction of regulatory cells mediating ACAID was abolished by in vivo blockade of CD80 and CD86. The present results indicated that CD80 and CD86 play a critical role in induction of ACAID, possibly by co-stimulating expansion and IL-10 production of regulatory T cells in response to TGF-β-conditioned APC.

Introduction

Various antigens, ranging from allografts to soluble heterologous proteins, injected into the anterior chamber (AC) of the eye elicit a uniquely deviated systemic immune response, termed anterior chamber-associated immune deviation (ACAID), which is characterized by the suppression of inflammatory T<sub>H</sub>1 responses, including delayed-type hypersensitivity (DTH) and production of complement-fixing antibodies (1-4). ACAID arises because intraocular antigen-presenting cells (APC), inducing macrophages and dendritic cells, capture antigens injected into the AC, migrate through the trabecular meshwork into the blood and then traffic preferentially to the spleen, where they induce antigen-specific regulatory T cells suppressing T<sub>H</sub>1 responses (3, 4).

Transforming growth factor-β (TGF-β), abundant in aqueous humor of the AC, plays a critical role in the induction of ACAID by conditioning the intraocular APC to acquire ACAID-inducing potential (5, 6). Thus, conventional APC such as macrophages in thioglycollate-elicited peritoneal exudate cells (PEC) can also acquire the ACAID-inducing potential after TGF-β treatment in vitro (6). We recently demonstrated that antigen presentation by the TGF-β-treated PEC stimulated naive T cells to produce IL-4 and IL-10, but not IFN-γ, possibly due to the inability to produce IL-12 (7, 8).

Activation and differentiation of T cells requires not only cytokines but also co-stimulatory molecules expressed on APC in addition to the engagement of antigen-specific TCR. CD28 is the most extensively characterized co-stimulatory receptor on T cells, which interacts with CD80 and CD86 on APC (9). In addition, some members of the tumor necrosis factor receptor superfamily, including CD27, OX40 and 4-1BB, also transmit a co-stimulatory signal for T-cell proliferation and cytokine production (10). In the present study, we determined the expression of CD80, CD86, CD27 ligand (CD70), OX40 ligand (OX40L) and 4-1BB ligand (4-1BBL) on the TGF-β-treated PEC and their role in co-stimulating the expansion and IL-10 production of T cells in vitro. We also
found a critical contribution of CD80 and CD86 to the induction of regulatory T cells mediating ACAID in vivo.

Methods

Animals and cell lines

Male BALB/c mice were purchased from Charles River Japan (Atsugi, Japan). Mice transgenic for the ovalbumin peptide (OVA257–264)-specific and I-A^k-restricted DO11.10 TCR on a RAG^2–/– background were generously supplied by S. Koyasu (Keio University, Tokyo, Japan) and bred and maintained in the Oriental Yeast Co. (Tokyo, Japan). All mice were 6- to 7-week old at the start of experiments and kept under specific pathogen-free condition during the experiments. The hybridomas producing mAbs against MHC class II were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mg/ml penicillin and streptomycin and 50 μM 2-Mercaptoethanol.

Antibodies

Purified anti-mouse CD16/32 (2.4G2) and CD3 (145-2C11) mAbs, FITC-conjugated anti-mouse CD4 (RM4-4), CD8 (53-6.7), B220 (RA3-6B2), CD11b (M1/70) and NK1.1 (PK136) mAbs, PE-conjugated anti-mouse CD80 (16-10A1), CD86 (GL1) and CD70 (FR70) mAbs, isotype-matched control mouse IgGs and PE-labeled streptavidin were purchased from BD Pharmingen (San Diego, CA, USA). Rabbit anti-asialo GM1 antibody was purchased from Wako Biochemicals (Osaka, Japan). Purified and biotinylated anti-mouse CD80 (RM80, rat IgG2a), CD86 (PO.3, rat IgG2b), CD70 (FR70, rat IgG2b), OX40L (RM134L, rat IgG2b) and 4-1BBL (TKS-1, rat IgG2a) mAbs were prepared as described previously (11–14). Control rat IgG was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Culture medium

RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U ml^−1 penicillin, 100 μg ml^−1 streptomycin, 1 × 10^−5 M 2-ME, 0.1% BSA, 1 μg ml^−1 iron-free transferrin, 10 ng ml^−1 linoleic acid, 0.3 mg ml^−1 Na2Se and 0.2 mg ml^−1 Fe(NO3)3 was used for the preparation of PEC and coculture of T cells and PEC.

Preparation of CD4^+ T cells

Spleens were removed from normal BALB/c mice or RAG^2−/− DO11.10 TCR-transgenic mice and pressed through nylon mesh to produce a single-cell suspension. RBCs were lysed with Tris–NH4Cl. T cells were enriched by passage through nylon wool column (Wako Biochemicals). CD4^+ T cells were purified by treatment with a mixture of hybridoma supernatants (M5/114, J11d, RA3-3A1 and 3.155), rabbit anti-asialo GM1 antibody (100 μg ml^−1) and low-tox rabbit complement (Cedarlane, Hornby, Ontario, Canada). Splenic T cells were purified by passage through nylon wool column (Wako Biochemicals) and the treatment with a mixture of hybridoma supernatants (anti-MHC class II, anti-HSA and anti-B220) and low-tox rabbit complement (Cedarlane). The purity was >95% CD4^+ as determined by flow cytometry.

Preparation of PEC

PEC were obtained from normal BALB/c mice that had received 2 ml of 4% thioglycollate (Sigma–Aldrich) intraperitoneally (i.p.) 3 days before and cultured in 24-well plates (1 × 10^6 per well) in the culture medium with or without 5 ng ml^−1 of porcine TGF-β2 (R&D Systems, Minneapolis, MN, USA). After overnight culture, plates were washed three times with the medium to remove TGF-β2 and non-adherent cells. Approximately 60% of the cells initially added remained adherent in the wells and were used in all subsequent experiments. More than 95% of these adherent cells were CD11b^+ as estimated by flow cytometry.

Co-culture of CD4^+ T cells with PEC

Purified CD4^+ T cells (1 × 10^5 per well) were co-cultured with TGF-β-treated or untreated PEC (1 × 10^6 per well) pulsed with anti-CD3 mAb (1 μg ml^−1) or OVA (100 μg ml^−1) in the presence or absence of 10 μg ml^−1 of anti-CD80 mAb, anti-CD86 mAb, anti-CD70 mAb, anti-FOX40L mAb or control rat IgG in 96-well flat-bottom plates. After 48 h, IFN-γ and IL-10 contents in the cell-free culture supernatants were measured by using ELISA kits (BD Pharmingen) according to the manufacturer’s instruction. Proliferative response was assessed after 48 or 72 h from the initiation by pulsing the cultures with [3H]thymidine (0.5 μCi per well) for the last 9 h.

Flow cytometric analysis

Cells (5 × 10^5) were first pre-incubated with unlabeled anti-CD16/32 mAb to avoid non-specific binding of mAbs to Fcγ receptor and then incubated with a saturating amount of FITC-, PE- and biotin-labeled mAbs. After washing with PBS twice, the cells were incubated with PE-labeled streptavidin for biotinylated mAb. After washing with PBS twice, the stained cells (live-gated on the basis of forward- and side-scatter profiles and propidium iodide exclusion) were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA, USA) and data were processed using the CellQuest program (BD Biosciences).

Induction of ACAID and DTH assay

Induction and evaluation of ACAID were performed essentially as described previously (15). Briefly, BALB/c mice received an inoculation of OVA (Sigma–Aldrich, 50 μg per 2 μl) dissolved in PBS into the AC of the right eye. Some groups of the mice received 0.25 mg each of anti-CD80 mAb and anti-CD86 mAb or 600 μg of control rat IgG on the same day. All these mice then received a subcutaneous injection of OVA (100 μg per 100 μl) emulsified in CFA (Life Technologies, Grand Island, NY, USA) into the nape of the neck 3 weeks later. After 1 week, the mice were challenged with an intradermal injection of OVA (200 μg per 10 μl) into the right ear pinnae. After 24 h, the ear swelling was measured using a micrometer (Mitutoyo, MTI Corporation, Paramus, NJ, USA).
Local adoptive transfer (LAT) assay

Development of regulatory T cells was tested in the LAT assay essentially as described previously (16). Briefly, splenocytes prepared from BALB/c mice that had received intraocular inoculation of OVA (50 μg per 2 μl) and i.p. administration of anti-CD80 mAb and anti-CD86 mAb (300 μg each) or control IgG (600 μg) 14 days before were prepared as the regulator cells. Effector cells were prepared from lymph node (LN) of BALB/c mice that had been subcutaneously immunized with OVA (100 μg per 100 μl) in CFA 7 days before. Stimulator cells were prepared by pulsing PEC with OVA (100 μg ml⁻¹) overnight. Effector cells (5 × 10⁶), stimulator cells (1 × 10⁶) and regulatory cells (5 × 10⁶) were re-suspended in 10 μl of HBSS and inoculated into the ear pinnae of naive BALB/c mice. Ear swelling was measured at 24 h. Spleen cells from naive mice were used as a negative control for regulatory cells.

Statistical analysis

Data were analyzed by using analysis of variance and t test. P values <0.05 were considered significantly different.

Results

Expression of co-stimulatory molecules on TGF-β-treated PEC

We first examined the expression of CD80, CD86, OX40L, CD70 and 4-1BBL on TGF-β-treated PEC by flow cytometry.

Co-stimulation of CD4⁺ T cells by CD80 and CD86 on TGF-β-treated PEC

We next examined the contribution of CD80, CD86, OX40L and CD70 expressed on untreated or TGF-β-treated PEC to the co-stimulation of naive T cells. Naive CD4⁺ T cells were purified from the spleen of normal BALB/c mice and stimulated with anti-CD3 mAb in the presence of untreated or TGF-β-treated PEC. Blocking mAbs against CD80, CD86, OX40L and CD70 were added to the culture to determine the contribution of these molecules. Proliferative response and production of IFN-γ and IL-10 were assessed after 48 h. Results of representative
Critical contribution of CD80 and CD86 to induction of ACAID

Given the critical contribution of CD80 and CD86 to the co-stimulation of T-cell expansion and IL-10 production by TGF-β-treated PEC in vitro (Figs 3 and 4), we next explored the involvement of CD80 and CD86 in the induction of regulatory cells by intraocular inoculation of antigen.

To further clarify the contribution of CD80 and CD86, we examined the effect of a combination of anti-CD80 and anti-CD86 mAbs (Fig. 3). When co-stimulated by untreated PEC, the combined anti-CD80/CD86 mAbs did not significantly inhibit the proliferative response and only partially inhibited the IFN-γ and IL-10 production (Fig. 3). In contrast, when co-stimulated by TGF-β-treated PEC, proliferation was markedly inhibited and IL-10 production was totally abolished by the combination of anti-CD80 and anti-CD86 mAbs (Fig. 3a and c). The similar contributions of CD80/86 on co-stimulation of TGF-β-treated PEC were also observed when TGF-β-treated PEC were pulsed with protein antigen and used as APC. Results of representative experiments are shown in Fig. 4. In this experiment, naive CD4+ T cells were purified from DO11.10 TCR-transgenic mice of which T cells possess OVA-specific TCR and stimulated with OVA-pulsed TGF-β-treated or untreated PEC. Proliferative response was assessed after 48 h and production of IFN-γ and IL-10 was assessed after 72 h. When DO11.10 TCR-transgenic T cells were stimulated with OVA-pulsed and untreated PEC, the combined anti-CD80/CD86 mAbs did not significantly inhibit the proliferative response, or the IL-10 production, and only partially inhibited the IFN-γ production (Fig. 4). In contrast, when DO11.10 TCR-transgenic T cells were stimulated with OVA-pulsed and TGF-β-treated PEC, both proliferative responses and IL-10 production were notably inhibited by the combination of anti-CD80 and anti-CD86 mAbs (Fig. 4a and c), which were compatible with the results of the experiments using anti-CD3 mAb.

These results indicated that the co-stimulation by untreated PEC was only partially dependent on CD80 and CD86, but that by TGF-β-treated PEC was predominantly mediated by CD80 and CD86.

Fig. 2. Involvement of CD80, CD86, OX40L and CD70 in co-stimulatory effect of untreated or TGF-β-treated PEC on anti-CD3-stimulated T-cell proliferation and cytokine production. Splenic CD4+ T cells were co-cultured with untreated or TGF-β-treated PEC in the presence of anti-CD3 mAb and the indicated mAbs for 48 h. Proliferative response was assessed by pulsing the cultures with [3H]thymidine for the last 9 h (a). IFN-γ (b) and IL-10 (c) contents in the cell-free supernatants were measured by ELISA. Data are expressed as the mean ± SD of triplicate samples. Similar results were obtained in three independent experiments. NS, not significantly different; *P < 0.05 for anti-CD80, anti-CD86, anti-OX40L or anti-CD70 mAb compared with control IgG. **P < 0.05 for untreated PEC compared with TGF-β-treated PEC described by lines.
cells by intraocular antigen inoculation in vivo. We employed
the LAT assay (16) to determine the development of regulatory
cells. LN cells from BALB/c mice that had been subcutane-
ously primed with OVA/CFA were used as the DTH effector.
OVA-pulsed PEC were used as the stimulator. Splenocytes
from BALB/c mice that had received an intraocular inoculation
of OVA and an i.p. administration of anti-CD80/CD86 mAbs or
control IgG 14 days before were used as the regulator. As
shown in Fig. 5, co-injection of OVA-primed LN cells as the
effector with OVA-pulsed PEC (stimulator) and naive spleno-
cytes (regulator) induced a substantial level of DTH response
as compared with naive LN cells as the effector. This DTH
response was significantly suppressed by the co-injection of
splenocytes from intraocularly OVA-inoculated and control
IgG-treated mice as the regulator. Importantly, this suppres-
sion was totally abrogated by the anti-CD80/CD86 mAb
treatment. These results suggested that CD80 and CD86
played a critical role in the induction of regulatory cells by
intraocular inoculation of OVA.

Critical contribution of CD80 and CD86 to induction
of ACAID

We finally examined the contribution of CD80 and CD86 to the
induction of ACAID by intraocular inoculation of antigen. Thus,
BALB/c mice were intraocularly inoculated with OVA and i.p.
administered with anti-CD80/CD86 mAbs or control IgG. Three
weeks later these mice were subcutaneously immunized with
OVA/CFA and then ear challenged with OVA 1 week later. As
shown in Fig. 6, ACAID was induced in the control IgG-treated
mice as manifested by a marked suppression of DTH
response. Notably, this suppression was totally abrogated in
the anti-CD80/CD86 mAb-treated mice. These results sug-
gested that CD80 and CD86 played an essential role in the
induction of ACAID by intraocular inoculation of OVA.

Discussion

In this study, we first compared the expression of co-
stimulatory molecules on TGF-β-treated PEC with that on
TGF-β-untreated PEC. While CD80 and CD86 were constitu-
tively expressed on freshly isolated CD11b+ PEC and not
affected by overnight treatment with TGF-β, OX40L and CD70
were down-regulated by the TGF-β treatment (Fig. 1). We
previously reported that the TGF-β treatment down-regulated
CD40 on PEC, resulting in the inability to produce IL-12 upon
interaction with CD40 ligand expressed on activated T cells
and thus to co-stimulate IFN-γ production by naive T cells (7).
Since both OX40L and CD70 were involved in the co-
stimulation of IFN-γ production by TGF-β-untreated PEC (Fig.
2b), the down-regulation of OX40L and CD70 might be also
responsible for the inability of TGF-β-treated PEC to co-
stimulate IFN-γ production.

![Fig. 3.](image) Critical contribution of CD80 and CD86 to co-stimulatory effect
of TGF-β-treated PEC on anti-CD3-stimulated T-cell proliferation and
IL-10 production. Splenic CD4+ T cells were co-cultured with TGF-β-
treated PEC in the presence of anti-CD3 mAb and anti-CD80 plus anti-
CD86 mAbs or control IgG for 48 h. Proliferative response was
assessed by pulsing the cultures with [3H]thymidine for the last 9 h (a).
IFN-γ (b) and IL-10 (c) contents in the cell-free supernatants were
measured by ELISA. Data are expressed as the mean ± SD of
triplicate samples. Similar results were obtained in three independent
experiments. *P < 0.05 compared with control IgG.
It is notable that co-stimulation by TGF-β-untreated PEC was largely CD80/CD86 independent (Figs 2–4). This might be due to redundant co-stimulatory functions of CD80/CD86, OX40L and CD70 expressed on the untreated PEC, since we previously demonstrated that OX40L and CD70 could co-stimulate naive T cells in a CD28-independent manner (17). Alternatively, some other co-stimulatory molecules, such as B7h engaging inducible co-stimulator (ICOS) on T cells (18, 19), might play a predominant role. Further studies are needed to address these possibilities.

In contrast to the TGF-β-untreated PEC, co-stimulatory function of TGF-β-treated PEC was mostly dependent on CD80 and CD86 (Figs 3 and 4). This seems to be due to the down-regulation of other co-stimulatory molecules by the TGF-β treatment (Fig. 1). Our present results indicate that TGF-β-treated PEC are not inert but actively induce the expansion and/or differentiation of IL-10-producing regulatory T cells via CD80/CD86 co-stimulation.

In ACAID, the intraocular injection of antigens prevents the DTH response, but enhances antibody production, to specific antigens. The results of a number of studies suggest that two types of regulatory cells, CD4+ and CD8+ T cells, exist in mice with ACAID, although each mediates its inhibitory effect on different stages of the immune response; the CD4+ population impairs the induction, whereas the CD8+ population inhibits the expression, phase of the DTH response (16, 20). Because mice with ACAID are unable to mount a DTH response to intraocularly injected antigen but do produce antibodies, it is possible that cells producing T1,2-type cytokines are induced, leading to a T1,2 cytokine-mediated down-regulation of the T1
indicating that the co-stimulatory function of TGF-β and the development of ACAID (Fig. 6). Given the inoculation inhibited the induction of regulatory cells (Fig. 5) and the expansion and/or differentiation of IL-10-producing regulatory T cells in response to ocular APC pre-conditioned by intraocular TGF-β, which mimic ACAID-inducing ocular APC, to induce proliferation and IL-10 production of naive T cells was mostly dependent on CD80 and CD86 (Figs 3 and 4), these results suggest that CD80 and CD86 play a critical role in the induction of ACAID by co-stimulating the expansion and/or differentiation of IL-10-producing regulatory T cells in response to ocular APC pre-conditioned by intraocular TGF-β.

It has been extensively characterized that CD80 and CD86 play a pivotal role in generating effector T-cell responses. Paradoxically, however, recent studies have revealed that constitutively expressed CD80 and CD86 play a pivotal role in generating effector T-cell responses. Paradoxically, however, recent studies have revealed that constitutively expressed CD80 and CD86 play a pivotal role in generating effector T-cell responses. Paradoxically, however, recent studies have revealed that constitutively expressed CD80 and CD86 play a pivotal role in generating effector T-cell responses.

**Fig. 6.** Involvement of CD80 and CD86 in induction of ACAID. BALB/c mice were injected with OVA intraocularly and anti-CD80 and anti-CD86 mAbs or control IgG i.p. Three weeks later these mice were immunized subcutaneously with OVA plus CFA. One week later their ear pinnae were challenged with OVA. Positive controls were immunized with OVA and CFA 7 days before the intrapinnae challenge with OVA. Naive mice were challenged with OVA as the negative control. Ear-swelling responses were measured 24 h after challenge. Data are represented as the mean ± SE of five mice in each group. Similar results were obtained in two independent experiments.

*P < 0.05.

**Abbreviations**

AC anterior chamber

AICAID anterior chamber-associated immune deviation

<table>
<thead>
<tr>
<th>Antigen-presenting cells</th>
<th>TGF-β transforming growth factor-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-1BB ligand</td>
<td>4-1BBLL</td>
</tr>
<tr>
<td>DTH delayed-type hypersensitivity</td>
<td></td>
</tr>
<tr>
<td>i.p. intraperitoneal</td>
<td></td>
</tr>
<tr>
<td>LAT local adoptive transfer</td>
<td></td>
</tr>
<tr>
<td>LN lymph node</td>
<td></td>
</tr>
<tr>
<td>2-ME 2-Mercaptoethanol</td>
<td></td>
</tr>
<tr>
<td>OVA ovalbumin peptide</td>
<td></td>
</tr>
<tr>
<td>OX40L OX40 ligand</td>
<td></td>
</tr>
<tr>
<td>PEC peritoneal exudate cells</td>
<td></td>
</tr>
</tbody>
</table>

**References**


