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NKT Cell-Derived Urokinase-Type Plasminogen Activator Promotes Peripheral Tolerance Associated with Eye

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In a model of peripheral tolerance called anterior chamber-associated immune deviation (ACAID), the differentiation of the T regulatory cells depends on NKT cells and occurs in the spleen. In this study, we show that NKT cells that express the invariant (i) TCR and are the CD1d-reactive NKT cells (required for development of peripheral tolerance) actually produced urokinase-type plasminogen activator (uPA) during tolerance induction. The RT-PCR and in vitro plasmin assay showed that splenic iNKT cells derived uPA-converted plasminogen to plasmin. Moreover, uPA was required for tolerance induction because uPA knockout (KO) mice did not develop peripheral tolerance or develop CD8\(^+\) T regulatory cells after Ag inoculation into the anterior chamber. In contrast, other aspects of ACAID-induced tolerance, including recruitment of iNKT cells to the spleen and production of IL-10 by iNKT cells, were unchanged in uPA-deficient mice. The adoptive transfer of splenic NKT cells from wild-type mice restored ACAID in iNKT cell-deficient, but NKT cells from uPA KO mice did not. We postulate that the mechanism of action of uPA is through its binding to the uPAR receptor, and enzymatic cleavage of plasminogen to plasmin, which in turn activates latent TGF\(\beta\). In conclusion, uPA derived from iNKT cells is required to induce peripheral tolerance via the eye. The Journal of Immunology, 2007, 179: 2215–2222.

Immune privilege in the eye is attributed to various local factors including the lack of lymphatic drainage (1), Fas ligand expression (2), and multiple immunosuppressive factors in aqueous humor (3). Importantly, the eye is immunosuppressed locally to reduce excessive intraocular inflammation but, through a process known as anterior chamber-associated immune deviation (ACAID), has the ability to induce peripheral tolerance (4).

ACAID is characterized by a selective deficiency in delayed hypersensitivity (DH) and Ig isotypes that fix complement (5, 6). Central to the ACAID process are intraocular bone marrow-derived F4/80\(^+\) APCs that capture Ag within the anterior chamber (a.c.) and carry an Ag-specific ACAID-inducing signal via the blood directly to the spleen (5, 7). Splenic generated CD8\(^+\) T regulatory (Treg) cells negatively regulate the effector phase of the DH response within 7 days of a.c. inoculation (8). ACAID CD8\(^+\) Treg cells are central to the development of the Ag-specific Treg cell (9, 11–13).

The peripheral tolerance is mediated by an afferent CD4\(^+\) Treg cell and an afferent CD8\(^+\) Treg cell (14–16). The mechanism of Treg suppression is not fully understood and may be mediated by soluble factors and cell-cell contact. Keino and colleagues report that ACAID CD4\(^+\) Tregs are different from natural CD4 Tregs, and ACAID CD4CD25\(^+\) Tregs express FOXP3 but ACAID CD4\(^+\)CD25\(^+\) Tregs do not. The assay used in these studies measures ACAID studies suppression of effector responses, and therefore results relate to the generation of CD8\(^+\) Treg cells.

NKT cells belong to a specialized population of lymphocytes that coexpress the TCR \(\alpha\beta\)-chain and NK markers (17, 18). A major subpopulation of NKT cells express a unique invariant V\(\alpha14\)J\(\alpha8\) Ag receptor not expressed by conventional T cells (17, 19–21). NKT cells are restricted by MHC class I-like CD1d molecules (22, 23), and because the CD1d molecule also is required for the development of NKT cells, CD1d knockout (KO) mice selectively lack the CD1d restricted NKT cells (24–26).

In the ACAID model, CD4\(^+\) invariant (i)NKT cells are recruited to the spleen by MIP-2 (27). Released from F4/80\(^+\) APC, iNKT cells interact with the CD1d molecule expressed by APC and blocking CD1d protein interfered with the development of ACAID (11). Following the NKT cell/CD1d interaction, CD1d-activated NKT cells produce RANTES, which in turn recruits precursor Treg CD8\(^+\) cells and more F4/80\(^+\) APC to the spleen (28), some of which come from the eye. The F4/80\(^+\) APC, NKT cells, and CD8 T cell aggregate with B cells (29, 30) in the splenic marginal zone (27). NKT cells are crucial for the development of ACAID and secrete IL-10 required for induction of mature Treg cells (31). However, the precise mechanism used by NKT cells to influence the development of Treg cells in the ACAID model is not fully understood. We propose that the NKT cell contributes to the immunosuppressive environment that in turn biases the T

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3 Abbreviations used in this paper: ACAID, anterior chamber-associated immune deviation; DH, delayed hypersensitivity; a.c., anterior chamber; Treg, T regulatory; KO, knockout; i, invariant; PA, plasminogen activator; iPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; PEC, peritoneal exudate cell; WT, wild type; LAT, local adoptive transfer.
cell differentiation toward T cell regulation rather than immune reactivity.

The plasminogen activator (PA) system is a general enzyme system that provides proteolytic activity in many biological processes involving extracellular matrix degradation, tissue remodeling, complement activation, and cell migration (32–34). The activation of plasminogen to the broad-spectrum protease plasmin is performed by either of the two physiological PAs, tissue-type PA (tPA) or urokinase-type PA (uPA). Activation of the PA system is initiated by the release of PAs from specific cells in response to external signals and results in local proteolytic activity (32, 33). Because of the high concentration of plasminogen in virtually all tissues, the production of relatively small amounts of PA can result in high local concentrations of plasmin (33). By acting in concert with other proteinases, plasmin has also been proposed to play a role in degradation of the extracellular matrix during many physiological and pathological processes such as ovulation (35), wound healing (36), angiogenesis (37), and so on. Moreover, it was demonstrated that uPA and uPAR play important roles in initiating and maintaining innate and adaptive immunity (33).

The NKT cell is known to produce TGFβ, but because most TGFβ is latent, another important function of the NKT cells would be to provide enzymes that might activate TGFβ. Because uPA was reported to be involved in both activation of TGFβ in a variety of systems and TGFβ is central to the induction of ACAID, we postulated that NKT cells were required for ACAID because they produced uPA.

In this report, we present a role for uPA in immune regulation and demonstrate that uPA produced by iNKT cells is absolutely required for the induction of Ag-specific Treg cells following the inoculation of Ag into the eye. Thus, this report shows a novel role for uPA, uPAR, and the plasminogen system in immune regulation and peripheral tolerance.

Materials and Methods

Mice

Female, 8- to 10-wk-old mice were used in all experiments. C57BL/6 (B6) mice were obtained from Taconic Farms. NKT KO mice (Ja18 KO mice) were generated at Chiba University (Chiba, Japan) (20) and backcrossed nine times to B6 mice (N9) and maintained in the Schepens animal facility from the original breeding pair received from M. Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama City, Kanagawa, Japan). uPA, uPAR, and plasminogen KO mice were generated at Vlaams Interuniversitair Instituut (Leuven, Belgium) and backcrossed six times to B6 mice (N6) (38).

Genotyping was performed as follows: Tail DNA was isolated using DNsase (Invitrogen Life Technologies) according to the manufacturer’s recommendation. Two to 3 μg of DNA was digested overnight with 20 U of EcoRV (Invitrogen Life Technologies) at 37°C in a 50-μl reaction. After overnight digestion, the DNA was subjected to electrophoresis on a 0.8% agarose gel. After electrophoresis and denaturation/neutralization of the gel, the DNA was transferred to Magnagraph nylon transfer membrane (Osmonics) overnight in 20X SSC. The DNA probe for analysis was labeled via random priming with [32P]dCTP, and hybridization was performed with 1 X 10⁶ cpm/ml in KPL formamide hybridization buffer for 24 h at 42°C. After hybridization, the blots were washed two times with 2X SSC for 10 min at 42°C followed by two washes with 0.2X SSC for 10 min at 42°C. Membranes were then exposed to Kodak X-OMAT X-ray film overnight at ~70°C.

The animals were maintained on food and water ad libitum until they reached the desired weight (20–24 g). All animals were treated humanely and in accordance with the Schepens Animal Care and Use Committee and National Institutes of Health guidelines.

Induction of immune deviation and assay for DH

ACAID was induced in mice by inoculating OVA (50 mg/2 ml in HBSS; Sigma-Aldrich) into the a.c. 7 days before sensitizing (s.c.) with OVA in CFA to induce DH response. Intravenously induced immune deviation was induced by inoculation of the Ag (OVA; 50 mg/100 ml in HBSS) into the tail vein with a 30-gauge needle 7 days before immunizing for DH. To induce DH, mice received a s.c. inoculation with OVA (100 mg/ml in HBSS; 50 μl) emulsified in CFA (50 μl) and a week later were tested for the development of DH by an intradermal inoculation of OVA-pulsed peritoneal exudate cells (PECs) (prepared as described below; 2 X 10⁵/10 ml HBSS) into the right ear pinnae. Ear swelling was measured 24 and 48 h later with an engineer’s micrometer (Mitutoyo; MTI).

Preparation of OVA-pulsed PECs

PECs were obtained from peritoneal washes of B6 mice 3 days after they received an i.p. inoculation of 2.5 ml of 3% aged-thioglycollate solution (Sigma-Aldrich). After counting, PECs were cultured with OVA (5 mg/ml) in a 24-well culture plate in serum free medium (RPMI 1640 medium, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin (BioWhittaker)) and supplemented with 0.1% BSA (Sigma-Aldrich) and ITD 2 culture supplement (1 mg/ml iron-free transferrin, 10 ng/ml linoleic acid, 0.3 ng/ml Na₂Se, and 0.2 mg/ml Fe(NO₃)₂ (Collaborative Biomedical Products). Nonadherent cells were removed from the cultures after 18 h by three washes and the remaining adherent cells were collected by vigorous pipetting with cold medium (4°C) before washing (three times with HBSS) to remove free OVA.

NKT/NK cell enrichment

NKT/NK cell enrichment was performed as previously described (31). In short, IMMULAN column-enriched splenic T cells were harvested from wild-type (WT) mice or IL-10 KO mice. Cells were treated with FITC-conjugated anti-NK1.1 mAb before magnetic beads selection. Ab-labeled cells were treated with anti-FITC MicroBeads (Miltenyi Biotec) for 15 min and washed twice. To harvest NK/NKT cell-enriched cells, cells were applied to type MS⁺ positive selection column with MiniMACS (Miltenyi Biotec). Positively selected cells were stained with CyChrome 5-conjugated anti-TCRβ chain mAb, and enrichment was confirmed by flow cytometry. The cell numbers of enriched populations were adjusted to approximate the number used in the control studies.

NKT cells were further enriched by sorting for cells expressing intermediate density of the TCRβ-chain and NK1.1 molecules by FACs (EPICS Cell sorter; Beckman Coulter) with >97% purity (33).

Reconstitution of Ja18 KO mice

Ja18 KO mice were gamma-irradiated (cesium, 200 rad; Mark 1 irradiator; J. L. Shepherd and Associates) 1 day before receiving 10⁶ NKT/NK-enriched cells from either WT mice or uPA KO mice by i.v. route. NKT/NK cells were obtained by passing whole splenocytes over IMMULAN columns (Biotecx Laboratory) to remove macrophages and B cells. Twenty-four hours after reconstitution, reconstituted NKT KO mice were inoculated (s.c.) with OVA (50 mg/2 ml in HBSS). Spleens were removed a week after the a.c. inoculation, dissociated cells were pooled, and naive T cells were enriched as described above. Enriched splenic T cells were transferred to naive B6 mice as regulator cells with effector (derived from B6 mice) and stimulator cells (derived from B6 mice) and tested in a local adoptive transfer (LAT) assay.

Local adoptive transfer

To test for the effector regulatory cell of ACAID, a modified LAT assay was performed as described elsewhere (14). In brief, T effector cells were generated in B6 mice by immunizing (s.c.) with OVA in HBSS, and CFA, and 7 days later the primed T cells were enriched from dissociated spleen cells by removing B cells and macrophages using IMMULAN columns (Biotecx Laboratory). Regulatory cells were similarly enriched on IMMUNAL columns from splenocyte columns of ACAID mice 7 days post a.c. inoculation of OVA. Stimulator cells were OVA-pulsed PECs as described below. Effector (5 X 10⁵), stimulator (5 X 10⁴), and regulatory (5 X 10⁴) cells were mixed and resuspended in 10 ml of HBSS for inoculation into the right ear pinnae of naive mice. Ear swelling was measured with an engineer’s micrometer at 24 and 48 h. As a negative control, naive T cells from unmanipulated mice were used as effector cells and regulator cells. Primed T cells were used as effector cells and naive T cells from unmanipulated mice were used as regulator cells for positive control.

Plasmin assay

Plasmin activity was measured using the chromogenic substrate Val-Leu-Lys-p-nitroanilide (Sigma-Aldrich). Enriched NKT cells (10⁵/50 μl PBS) were incubated with a constant concentration of plasminogen medium (50
μl) for 3 hours in the 96-well plate and added 0.6 mM substrate in 50 mM Tris-HCl (pH 7.4) and 110 mM NaCl. The absorbance at 405 nm was recorded. Backgrounds due to overlapping activities of other serine proteases were determined using skin extracts plus the plasmin inhibitor 2-AP.

Plasmin activity was expressed as relative plasmin activity (OD$_{405}$/mg protein in the presence of 2-AP). For positive control, we used in vivo Con A-stimulated splenic NKT cells. NKT cells were harvested as described above from the Con A (10 mg/kg)-treated mice 12 h ahead.

**Antibodies**

The Abs used for flow cytometry analysis were as follows: Fc Block (anti-mouse FcγRI/II/IId mAb, 2.4G2), biotin- or FITC-conjugated anti-NK1.1 mAb (PK136), and CyChrome 5-conjugated anti-TCRβ chain mAb. The previous Abs were purchased from BD Pharmingen. Streptavidin-PE was purchased from Jackson ImmunoResearch.

**Flow cytometry**

Splenic NK and NKT cells were analyzed by flow cytometry. RBC were lysed by adding Tris-buffered ammonium chloride to a cell pellet of spleen cells. Staining was performed in the presence of a saturated concentration of Fc Block (blocks FcγRI/II/IId). Cells were stained with the following reagents and colors (using concentrations recommended by the manufacturer): biotin-conjugated anti-NK1.1 mAb counterstained with streptavidin-PE; CyChrome 5-conjugated anti-TCRβ chain mAb. Stained cells were analyzed on an EPICS XL flow cytometer (Beckman Coulter). The absolute number of splenic NKT cells detected in flow cytometry was calculated from the percentage of NKT cells in the number of viable cells. The total number of viable cells harvested from the spleens before staining was determined by the trypan blue exclusion method.

**RT-PCR**

Total RNA were extracted from whole splenocytes and enriched NKT cells 7 days after a.c. inoculation using TRIzol. To identify β-actin and IL-10 mRNAs, total RNA was reverse-transcribed and amplified by the Access RT-PCR System (Promega) according to the manufacturer’s guidelines. PCR products were electrophoresed on 1.8% agarose gel in the presence of 100,000× GelStar nucleic acid gel stain (FMC BioProducts). Bands were photographed and quantified by FX Molecular Imaging System (Bio-Rad Laboratories). The amount of RNA in each sample was standardized by preliminary amplification for β-actin, and readjusting the sample concentration according to densitometry reading of β-actin bands, as described above. The adjusting systems were repeated until β-actin bands were equalized in serially diluted samples.

Primers used in these experiments are listed below: for amplification: β-actin, sense, 5′-TGGAAGACATGAGTGCAGCACTGAAGCAGC-3′, and antisense, 5′-CTCCATCCTGTTTGCTGGAAGATAGCAGGAC-3′; and antisense, 5′-CTAACGTAGGAGAGATGAGCTGTCGAAGACAG-3′; and antisense, 5′-CTAACTGCTGTCAGGACAGCAGCAGCAAG-3′; and antisense, 5′-AGGAGACTGCACCATTTT-3′; and antisense, 5′-AAGACCACCATTTT-3′. (product size, 313 bp).

**Statistics**

Data were analyzed for significant differences among experimental groups by ANOVA and post hoc Scheffe’s test. A value of p ≤ 0.05 was considered significant.

**Results**

**NKT cell-derived uPA is produced during development of ACAID**

After Ag is inoculated into the eye, the development of peripheral tolerance, in general, and Ag-specific Treg cells, in particular, is dependent on NKT cells interacting with CD1d molecules (11). Thus, we proposed that the CD1d interaction stimulated the NKT cell to produce multiple factors that could influence the differentiation of T cells into regulatory cells. Because uPA and NKT cells are known to convert latent TGFβ to active TGFβ, we tested to see whether an increase in uPA in RNA expression in the splenic iNKT cells correlated with the development of peripheral tolerance.

Although early reports showed that TGFβ is required for the development of ACAID, most TGFβ in the body is latent and it must be separated from the latent binding protein if it is to bind its receptor and be effective. We reasoned that a possible role for NKT cells in ACAID might be to produce an enzyme capable of converting latent TGFβ to its active form.

To examine whether NKT cell production of uPA was unique to peripheral tolerance induced via the eye, we compared the uPA mRNA measured on samples from a.c.- or i.v.-inoculated WT (B6) mice. The experiment was repeated three times with similar results. B. Left: Purified NKT cells (1 × 10^5) were added to plasminogen containing medium in the 96-well plate, and 3 h later, the concentration of plasmin was measured. The concentration is indicated in microunits per milliliter on the ordinate. The treatment of the different experimental groups is shown along the abscissa. Right, Purified NKT cells from Con A-pretreated mice were used as positive control.

![FIGURE 1. RT-PCR measurement of uPA mRNA in purified splenic NKT cells. A. Total RNA was extracted from purified splenic NKT cells from naive, a.c.- or i.v.-inoculated WT (B6) mice. Left panel shows bands on the agarose gel for uPA and β-actin (indicated with arrows). Right panel shows a bar graph of the ratio of densitometer readings for uPA mRNA. The experiment was repeated three times with similar results. B. Left, Purified NKT cells (1 × 10^5) were added to plasminogen containing medium in the 96-well plate, and 3 h later, the concentration of plasmin was measured. The concentration is indicated in microunits per milliliter on the ordinate. The treatment of the different experimental groups is shown along the abscissa. Right, Purified NKT cells from Con A-pretreated mice were used as positive control.](http://www.jimmunol.org/-/media/jimmunol/2007/0015906/015906-f1.jpg)
NKT cells from a.c.-inoculated uPA KO mice (that contained tPA) were unable to produce equivalent levels of plasmin as NKT cells from WT mice. We concluded that NKT cells derived from a.c.-inoculated mice produce uPA and convert plasminogen to plasmin.

uPA KO mice fail to develop ACAID

Thus far, we show that an increase in uPA mRNA, and uPA function in NKT cells are correlated with the induction of ACAID. To test whether the uPA is required for ACAID development, we induced ACAID in uPA KO and WT mice. WT and uPA KO mice received OVA (a.c.) inoculation 7 days before they received an immunizing dose of the Ag and CFA (a.c.). As before, 7 days later, they were challenged in the ear pinnae with Ag or Ag-pulsed APC. Change in ear thickness measurements was used as an indication of a DH challenged with Ag response (24 h later). The induction of ACAID in WT mice, but not in the mice lacking uPA caused a suppression of DH (Fig. 2). These data show that uPA participates in the development of DH suppression and peripheral tolerance.

uPA KO mice fail to develop Treg cells in ACAID

An important mediator of peripheral tolerance in ACAID is the efferent CD8⁺ Treg cell. To test whether the lack of DH suppression correlated with a defect in the Ag-specific, efferent Treg cells, we used a LAT assay (11, 31). uPA KO mice were inoculated (a.c.) with OVA 7 days before harvesting, dissociating, and enriching T cells from the spleens (see Fig. 5A). The cells were transferred to Ja18 KO mice to reconstitute NKT cells, and then the reconstituted Ja18 KO mice were a.c.-inoculated with OVA. A LAT assay was performed to examine the Treg cell induction ability. Regulatory cells were cotransferred with responder and stimulator cells into naive recipient mice (n = 5). Mixtures of cells injected into the ear pinnae are indicated along the ordinate below each bar. Changes (Δ) in ear swelling measurements (24 h after ear challenge) are shown on the ordinate. Significant differences (p < 0.05) are indicated by an asterisk (*). The experiment was repeated three times with similar results.
NKT cells from uPA KO mice could not reconstitute ACAID in NKT KO

To address the question of whether the uPA was derived from NKT cells, we used NKT cell-deficient mice (J/H9251 18 KO) as recipients of WT and uPA KO NKT cells. This experiment tested the requirement of NKT cell-derived uPA in the reconstitution of ACAID in the iNKT cell-deficient mouse (11). Seven days after NKT/NK cells (10^6) enriched from spleens of uPA KO (or WT) mice were transferred (i.v.) into J/H9251 18 KO mice. The reconstituted and nonreconstituted deficient mice were inoculated with OVA. A week later, T cells were enriched from the spleens and used as potential Treg cells in the LAT assay. Control regulatory cells were enriched T cells from spleens of a.c.-inoculated naive (irradiated) J/H9251 18 KO mice. As expected, Treg cells from a.c.-inoculated WT mice suppressed the DH response in the ear (Fig. 4). However, NKT/NK-enriched spleen cells from uPA KO mice did not (Fig. 4). Thus, we conclude that NKT cell-derived uPA is essential for the differentiation of Treg cells during peripheral tolerance induction subsequent to a.c. inoculation of OVA.

uPA deficiency does not interfere with NKT cells accumulation in the spleen or their ability to produce IL-10 after a.c. inoculation

Because uPA has multiple biological functions, the interference with the development of peripheral tolerance development could occur at various biological steps. Previously, we demonstrated that the NKT cells accumulated in the spleen after a.c. inoculation of Ag produced IL-10 ACAID (31). To clarify whether the deficiency of ACAID-inducing ability in uPA KO mice was due to the role of uPA in the increase in NKT cell numbers in the spleen or the ability of the NKT cell to produce IL-10 after a.c.-inoculated mice, we induced ACAID in WT and uPA KO mice.

First, WT (B6) mice and uPA KO mice were inoculated (a.c.) with OVA, and 7 days later the spleens were extirpated, cells dissociated, and the numbers of NKT cells were analyzed by flow cytometry after staining for the TCR α-chain and the NK1.1 molecule. Analyses were performed on five individual mice per group. The absolute number of splenic NKT cells detected in flow cytometry was calculated from the percentage of NKT cells in the number of viable cells. The absolute number of NKT cells in the spleen was increased in both B6 mice and uPA KO mice compared with naive animals (Fig. 5A). Thus, uPA is not needed for migration of NKT cells to the spleen in ACAID. Furthermore, this increase in NKT cells was not dependent on the Abs used to gate or select the NKT cells, because similar increases in NKT cells after a.c. inoculation Ag are seen if the T cell gate were analyzed with J/H9251 18-galactosylceramide-loaded dimers vs NK1.1 Ab (data not shown).

To determine relative quantities of the IL-10 mRNA, we used RT-PCR to analyze the spleens of similarly treated mice. The IL-10 mRNA levels in spleen cells from untreated mice were compared with those of a.c.-inoculated WT mice or uPA KO mice. Analysis of RNA from a.c.-inoculated B6 mice and a.c.-inoculated
uPA KO mice, clearly showed an increase in IL-10 mRNA compared with RNA from naive mice (Fig. 5B). Therefore, the inability of uPA KO mice to develop Treg cells and ACAID is not due to a lack of NKT cell-derived IL-10.

A role for the plasminogen system in ACAID

uPA has been implicated in a number of biological paradigms in addition to its enzymatic function (33). Plasmin is efficient in the enzymatic activation of latent TGFβ. To explore the role of the plasminogen system in ACAID, we inoculated Ag into the a.c. of plasminogen KO mice, uPAR KO mice, and WT mice. Following the deliberate sensitization of non-a.c.-inoculated mice and mice missing either the uPAR (Fig. 6A) or plasminogen (Fig. 6B) gene, there was a robust DH response. The WT mice that received the a.c. inoculation exhibited a suppressed DH response. These data support the conclusion that plasminogen, plasmin, and uPAR are required for the proper induction of ACAID, presumably for their function in the activation of latent TGFβ (Fig. 7).

Discussion

In this report, we show that splenic NKT cell-derived uPA (not tPA) is required for the induction of peripheral tolerance following the inoculation of Ag into the eye. Because NKT cells produce a variety of cytokines in response to signals (18), deficient uPA might interfere with the production of a critical cytokine. However, this report also shows that NKT cell-derived IL-10 (an essential cytokine for the induction of ACAID) is not dependent on uPA (31), because uPA KO mice adequately produced IL-10 during the process of tolerance induction. Thus, the uPA and IL-10 requirements in induction of peripheral tolerance via the eye are independent of each other and may be required at different phases of the tolerance process.

uPA is a serine protease that catalyzes the conversion of plasminogen to plasmin (33). uPA is secreted by many cell populations, including neutrophils and endothelial and epithelial cells, as a single-chain proenzyme (pro-uPA) that possesses little or no proteolytic activity (39, 40). The uPA and its receptor, uPAR, exert pleiotropic functions over the course of both physiological and pathological processes (33). In addition to its role in facilitating the cleavage of plasminogen to plasmin, uPA also has a key role in fibrinolysis, but also can modulate the development of protective immunity (41, 43). In this study, we show that in peripheral tolerance induced via the eye, the uPAR and plasminogen are critically involved and mice deficient in either are unable to develop peripheral tolerance to an a.c.-injected Ag.

Although the precise mechanisms of how eye-induced tolerance is mediated through NKT-derived uPA are currently unknown, this report supports a role for uPA in the conversion of plasminogen to plasmin. We postulate that NKT cell-derived uPA may convert existing latent TGFβ to its active form through plasmin (44).
propose that, once secreted, the NKT cell-derived uPA binds uPAR on the nearby F4/80 APC in the marginal zone where it converts plasminogen to plasmin. Plasmin is efficient at converting latent TGFβ to active TGFβ (Fig. 7). TGFβ is unusual among cytokines in that it is usually found in a biologically inactive form as a complex with latent TGFβ-binding protein. To express its biologic activity, TGFβ must be enzymatically dissociated from latent TGFβ-binding protein, thus liberating biologically active TGFβ (45). Because of this, the regulation of TGFβ activity is complex and occurs not only at the level of TGFβ production but also at the level of TGFβ activation. A role for plasmin activation of TGFβ is supported by a report from Bickerstaff et al. (46) that demonstrated that DH regulation in drug-induced cardiac allograft acceptor mice involved plasmin activation of TGFβ. Our preliminary data in bioassay using TGFβ-sensitive Mv1Lu cells showed that NKT cells derived from a.c.-inoculated mice were able to convert latent TGFβ to active (but not NKT cells from a.c.-inoculated uPA KO mice). TGFβ is a well-known immunosuppressive cytokine that is operative in several experimental models of immunity including ACAID (29, 47, 48).

uPA most likely contributes to the ad hoc immune-privileged environment that is set up in the spleen after the induction of peripheral tolerance via a.c. inoculation. In brief, it is known that F4/80 APC carry the eye-inoculated Ag to the spleen during ACAID induction (4) and that the eye-derived APC secretes MIP-2 (27), which in turn selectively recruits iNKT cells (49) to the marginal zone (50) where they aggregate F4/80 APC (27), B cells (29, 51, 52), and conventional T cells (28). Because there are essentially no NKT cells in the eye and splenocyte abolishes ACAID (13), we favor the postulate that the NKT cell-derived uPA that is required for ACAID via the plasminogen system functions (Fig. 7) within the marginal zone of the spleen and most likely between the cells that are aggregated.

Although not studied here, others have reported the uPA-uPAR system may contribute to bridge formation necessary to maintain cell-to-cell interactions. Such a role could also be contributing to cellular communication during the generation of Ag-specific T cells in ACAID. Previously, we reported that NKT cells, eye-derived F4/80 cells, and others formed the cluster in the splenic marginal zone 5 days after a.c. inoculation (27). Because uPA-uPAR complex is needed for β2 integrin-dependent leukocyte recruitment into the site of Ag presentation (53) the NKT cell-derived uPA interaction with uPAR on the F4/80 APC could be essential in the maintenance of the cell aggregates in the splenic marginal zone during ACAID induction. It is known that uPA anchors uPA at a cell surface and, in addition to favoring extracellular matrix degradation, regulates cell migration, cell adhesion, and cell proliferation; thus influencing the development of inflammatory and immune responses (33). The uPA-uPAR interactions can also influence cell behavior independent of any proteolytic activity. Additional nonenzymatic activities of the uPA-uPAR complex include changes in gene expression, signaling events, and cell-matrix adhesion (33).

Induction and maintenance of Ag-specific tolerance are required for immune homeostasis, prevention of autoimmune disorder, and the goal of allotransplantation. A relationship among ACAID, self-tolerance, and autoimmune is suggested by reports that induction of ACAID in mice both prevented the onset, and the expression of existing experimental autoimmune uveitis (54). Moreover, several published reports imply a role for NKT cells in preventing certain autoimmune disease in humans (55, 56). Also, our laboratory reported that transfer of in vitro-generated tolerogenic APC (that are known to induce Ag-specific tolerance in an NKT cell-dependent manner) has reduced the clinical symptoms of experimental auto-

immune encephalomyelitis (57), pulmonary interstitial fibrosis, and airway hyperreactivity in mice (10, 58). Together, these reports support the notion that autoimmunity associated with NKT cell defects may be mediated in part by disruption of organ-specific tolerance mechanisms due to NKT cell-derived uPA.

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References


