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Urokinase-Type Plasminogen Activator Is Required for the Generation of a Type 1 Immune Response to Pulmonary Cryptococcus neoformans Infection

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Urokinase-type plasminogen activator (uPA)−/− mice cannot mount protective host defenses during infection with the opportunistic yeast Cryptococcus neoformans (52D). Because effective host defense against C. neoformans requires specific immune responses and the generation of type 1 (T1) cytokines, we determined how the absence of uPA impacts these processes. Wild-type (WT) and uPA−/− mice were inoculated with C. neoformans. Macrophage antifungal activity was assessed histologically, T lymphocyte responses in vivo and proliferation in vitro were quantitated, and cytokine concentrations were determined by ELISA. uPA−/− macrophages have impaired antimicrobial activity. Regional lymph nodes of infected uPA−/− mice contained fewer cells than WT, suggesting impaired T cell proliferation in response to the pathogen in vivo. In vitro, uPA−/− T lymphocytes had impaired proliferative responses to C. neoformans rechallenge compared with WT. Infected WT mice generated T1 cytokines in the lung, characterized by high levels of IFN-γ and IL-12. uPA−/− mice had decreased levels of IFN-γ and IL-12, and increased IL-5, a type 2 cytokine. In the absence of uPA, the cytokine profile of regional lymph nodes shifted from a T1 pattern characterized by IFN-γ and IL-2 to a weak, nonpolarized response. We conclude that in the absence of uPA, lymphocyte proliferative responses are diminished, and mice fail to generate protective T1 cytokines, resulting in impaired antimicrobial activity. This study provides novel evidence that uPA is a critical modulator of immune responses and of immune cell effector functions in vivo. The Journal of Immunology, 2002, 168: 801–809.

T he expression of proteases is thought to be critically important for the ability of cells to degrade matrix proteins and traverse tissue planes during recruitment to inflammatory sites (1–3). Evidence implicates the urokinase-type plasminogen activator (uPA)/plasmin system as a central mediator in this process (4, 5). Leukocytes express uPA that converts the inactive proenzyme plasminogen to plasmin, a protease of broad substrate specificity (6). Leukocytes also express specific cell surface receptors for uPA, the urokinase receptor (uPA receptor (uPAR), CD87) (7–9).

Both mononuclear phagocytes (macrophages (Mφ) and neutrophils express uPA constitutively, and substantially up-regulate uPA expression in response to activating stimuli. By contrast, only activated lymphocytes express uPA (7, 10, 11). Thus, in lymphocytes uPA is a strict activation Ag, which suggests that uPA is intrinsically involved in the process(es) by which lymphocytes become activated.

In vivo investigation of the role uPA plays in pulmonary host defenses has previously been limited by the inability to completely and irreversibly eliminate uPA. This limitation has been overcome by the development of transgenic mice lacking the uPA gene (12). To determine whether uPA is required for effective pulmonary immune responses, we have established a murine model of pulmonary Cryptococcus neoformans infection (13). This model is well suited for the study of immune competency for several reasons. First, C. neoformans rarely causes disease in the immunocompetent, but is an important lethal fungal pathogen in immunocompromised patients and therefore has clinical relevance; second, C. neoformans strain 52D is not lethal in immunocompetent resistant mice but disseminates and causes death by meningitis when mice are immunocompromised (14, 15), paralleling the pattern of host susceptibility seen clinically; and third, host defense against C. neoformans is dependent on intact cell-mediated immunity (16).

We have previously demonstrated that uPA is required for protective pulmonary defenses against C. neoformans. In the absence of uPA, recruitment of inflammatory and immune cells in response to pulmonary C. neoformans infection is markedly diminished and the infection is not adequately combated; fungal CFUs in the lungs increase unchecked, and the infection disseminates to spleen and brain. The uPA−/− animals do not survive, and they die of fungal meningitis by day 85 post-intratracheal (IT) inoculation. In contrast, the wild type (WT) survive and clear the infection completely (17).

The requirement for intact cell-mediated immunity against C. neoformans is demonstrated clinically in HIV-infected patients whose incidence of C. neoformans infection increases dramatically
as they become CD4 lymphocyte deficient (16). Similarly, athymic nude mice and mice that are CD4 lymphocyte-depleted have markedly increased susceptibility to C. neoformans infections (14, 15). More recent work has shown that several specific cytokines are of particular importance in protective host defenses against C. neoformans. IFN-γ activates alveolar macrophages, increasing uptake of the fungus and inhibition of its growth in vitro, and in vivo, anti-IFN-γ Abs blocked lung clearance of C. neoformans in immunocompetent mice (18, 19). IL-2 has been shown to increase anticytotoxic activity of PBMCs (20). Collectively, these data have led to an appreciation of a requirement for the generation of a type 1 (T1) profile of cytokines for protective host defense against C. neoformans, characterized by the above-mentioned cytokines in addition to IL-12 (21).

uPA-deficient transgenic mice are not CD4 lymphocyte-depleted and actually have increased numbers of pulmonary CD4 lymphocytes at baseline compared with WT mice (17). Despite this, uPA−/− mice lack protective host defense against C. neoformans and behave like lymphocyte-depleted animals. This raises the possibility that the immune defect in the uPA−/− mice in response to pulmonary C. neoformans infection is not due only to a defect in cell number but also to a defect in cellular effector functions. In this study, we sought to determine whether the presence of uPA is required for the generation of a protective T1 profile of inflammatory cytokines in response to pulmonary C. neoformans infection.

Materials and Methods

Animals

Mice were housed in specific pathogen-free isolation rooms in the University of Michigan Department of Laboratory Animal Medicine (Ann Arbor, MI), which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. This study was approved by the University of Michigan Committee on Use and Care of Animals. Mice were periodically checked for murine hepatitis virus and were found to be negative, and were fed standard animal chow (rodent lab chow 5008; Purina, St. Louis, MO) and chlorinated tap water ad libitum. Mice were used at 8–12 wk of age.

Transgenic uPA-depleted mice (uPA−/−) and background-matched control mice uPA+/+ (WT) were generous gifts from P. Carmeliet (Center for Transgene Technology and Gene Therapy, Leuven, Belgium) (12). The uPA gene was knocked out by homologous recombinant with the uPA/neomycin construct in embryonic stem cells derived from strain 129 mice. The embryonic stem cells were injected into C57/B6 blastocysts, and the resulting chimeric males were bred with C57/B6 females to produce transgenic mice heterozygous for the uPA gene. Breeding of these progeny produced the homozygous mice used to establish the uPA−/− and uPA+/− lines used to generate colonies. Mice of this background (C57/B6/129) are immunocompetent and have preservation of complement-dependent acute lung injury (22). WT mice of this background (C57/B6/129), inoculated with the same strain of C. neoformans, were used in this study, are resistant to the infection—the mice survive, the fungus does not disseminate to brain, and the organism is cleared (17); furthermore, they have been shown to generate T1 cytokine responses (23) similar to those described in WT mice in this work.

Confirmation of genotype of uPA−/− and WT mice

We confirmed the genotype of the uPA−/− and WT mice by PCR analysis, as previously described (17). Genomic DNA was extracted from tail samples by proteinase K digestion, followed by chloroform extraction and ethanol precipitation (24). Samples were amplified with primers specific for the WT uPA gene (sense, 5′-ctgtctgcatccaaggct; antisense, 5′-gacacttgcacctttct; 25) or the knockout uPA/neomycin construct (sense, 5′-ctgtctgcatccaaggct; antisense, 5′-ctgtctgcatccaaggct; sequence pMC1Neo from Stratagene, La Jolla, CA), designed with the aid of the program Primer 2 (Scientific and Educational Software, Stateline, PA). Reaction mixtures (0.02 mg/ml DNA; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 2.5 mM MgCl2; 0.25 mM each nucleotide [dCTP, dATP, dTTP, dGTP]; 0.2 mM sense and antisense primers; 0.025 U/ml Taq polymerase; [Life Technologies, Gaithersburg, MD]) were denatured (94°C for 3 min) and amplified through 35 cycles (93°C for 30 s, 62°C for 45 s, 72°C for 120 s) with a one-cycle extension of 72°C for 5 min. Products were electrophoresed in agarose, stained with ethidium bromide, Southern blotted, and hybridized with labeled gene-specific internal oligonucleotide probes (26) to check product length and sequence specificity. Amplification products of the WT and knockout genes are 1153 or 1292 bp, respectively.

C. neoformans

C. neoformans strain 52 D was obtained from the American Type Culture Collection (Manassas, VA) (ATCC 24067; serotype D) (27). Stock cultures of C. neoformans were passed monthly on Sabouraud’s dextrose agar slants (Difco, Detroit, MI) and stored at 4°C. For infection, yeast was grown from stock in Sabouraud’s dextrose broth medium (1% peptone, 2% dextrose; Difco) for 48 h at 35°C on a shaker, washed twice in nonpyrogenic saline (NPS, Travenol, Deerfield, IL), counted on a hemocytometer, and diluted to 3.3 × 107 organisms/ml in NPS.

IT inoculation

Mice were lightly anesthetized with pentobarbital (64 mg/kg i.p.; Butler, Columbus, OH) and restrained on a small board. Each mouse received an IT inoculum of 1 × 108 C. neoformans in 30 μl of NPS, as previously described (28). This technique results in a highly reproducible pulmonary infection (13). Aliquots of the inoculum were serially diluted and plated out to confirm the number of CFU of C. neoformans being delivered. Control mice that received an IT inoculation with normal saline were examined histologically at various times after inoculation and showed no evidence of pulmonary inflammation.

Thin section histology and transmission electron microscopy

The lungs were perfused, the trachea cannulated, and the lungs inflated in situ with 2.5% gluteraldehyde in PBS. Next, the entire thoracic contents were dissected and fixed by immersion in 2.5% gluteraldehyde in PBS. Parasagittal sections through the fixed lungs were cut, dehydrated, embedded in Polybed 812 resin (Polysciences, Warrington, PA), and sectioned at 1-μm thickness. The slides, each representative of both lungs from a single mouse, were stained with toluidine blue. For transmission electron microscopy, samples were prepared as above, stained en bloc with 20% uranyl acetate in water, sectioned at 90 nm, poststained with saturated uranyl acetate and lead citrate, and examined in a LEO EM 900 (Leo, Thornwood, NY).

C. neoformans-stimulated proliferation

In separate experiments, 10 days after IT inoculation with C. neoformans, mice were euthanized, and the hilar and mediastinal lymph nodes were collected, disaggregated to a single cell suspension, counted, and cultured at 2 × 106 cells/well/200 μl of medium in 96-well culture plates (Costar, Cambridge, MA) in the presence and absence of heat-killed C. neoformans. Controls included nonstimulated lymph node cells that were harvested from unprimed WT and uPA−/− mice and cultured, as described above, in the presence and absence of heat-killed C. neoformans. The cells were cultured for 3 days. Eighteen hours before harvesting (PHD Cell Harvester; Cambridge Technology, Watertown, MA), the cells were pulsed with [3H]thymidine (1 μCi/well; Amersham, Arlington Heights, IL). Proliferation, as indicated by thymidine incorporation, was measured in counts per minute on a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA).

Cytokine determination by ELISA

At various times after inoculation, mice were killed with an overdose of pentobarbital. The lungs were subjected to bronchoalveolar lavage (BAL) by repeated instillation and removal of aliquots of PBS to a total volume of 1.5 ml. Regional lymph nodes (hilar and mediastinal) were removed, disaggregated to a single cell suspension, counted, and cultured at 2 × 105 cells/ml in NPS in 30-well culture plates (Costar, Cambridge, MA). Cytokine concentrations in the BAL fluid and in lung cellconditioned media were measured by ELISA (picograms per milliliter), using specific Ab pairs (BD PharMingen, San Diego, CA).

Adaptive transfer

Three weeks after IT inoculation with C. neoformans, donor WT mice were sacrificed. The lungs and regional lymph nodes were harvested and processed to a single cell suspension, as described above, and the nonadherent...
cells were placed in a prewarmed nylon wool column (Robbins Scientific, Mountain View, CA) and incubated at 37°C for 1 h. The nylon-wool non-adherent cells were slowly eluted with warm media, washed, pelleted, and resuspended in sterile PBS. A total of 2 × 10⁷ cells in 0.2 ml of PBS was infused via the tail vein into each recipient uPA⁻/⁻ mouse.

Assessment of DTH
Cryptococcal filtrate Ag was prepared as previously described (28). Mice were challenged with footpad injections of 30 µl of cryptococcal filtrate Ag in the right footpad and diluent in the left footpad. Forty-eight hours after injection, footpad thickness was measured by a micrometer (Lux Scientific Instrument, New York, NY) and expressed in micrometers. The thickness of the right footpad minus the thickness of the left footpad represents footpad swelling.

Statistical analysis
Comparisons between group means were performed by an unpaired Student’s t test. Statistical calculations were done using StatView 4.5 software (Abacus Concepts, Berkeley, CA). Data are expressed ± SEM. Statistical difference was accepted at p ≤ 0.05.

Results
Histologic comparison of WT and uPA⁻/⁻ alveolar Mφ
We previously demonstrated that uPA⁻/⁻ mice are unable to clear pulmonary C. neoformans infection and that the number of pulmonary inflammatory and immune cells are strikingly reduced in uPA⁻/⁻ compared with WT mice during the first 3 wk of infection. However, histologically, at late time points, the uPA⁻/⁻ mice had large numbers of alveolar Mφ (17). Despite this, uPA⁻/⁻ mice did not clear the pathogen. Therefore, we compared the histology of WT and uPA⁻/⁻ mice at day 35 post-IT inoculation. This time was chosen because it is a point in which we would expect to see the result of effective Mφ activation (microbial killing) or a lack of effective Mφ activation (microbial growth) clearly. We performed histologic studies using plastic imbedding and thin sectioning, and transmission electron microscopy. Demonstrated in Fig. 1A, by day 35 the WT lungs show relatively few alveolar Mφ. Most Mφ do not contain intracellular C. neoformans organisms, and scattered C. neoformans ghosts, appearing as empty, nonviable cell capsules, were present within the alveolar spaces. By contrast, the lungs of uPA⁻/⁻ mice (Fig. 1B) have alveolar spaces nearly filled with Mφ, many of which have the appearance of multinucleated giant cells. These Mφ have obviously ingested C. neoformans, many containing more than three organisms. Strikingly, however, as demonstrated by transmission electron microscopy in Fig. 1C, although the uPA⁻/⁻ Mφ are able to ingest C. neoformans, they have impaired intracellular killing. Not only are there viable C. neoformans present intracellularly, but the C. neoformans organisms are actively proliferating, as demonstrated by copious budding off the parent fungi within the Mφ. Thus, during pulmonary C. neoformans infection, uPA-deficient mice have impaired antimicrobial activity even when pulmonary Mφ number appears to be sufficient for host defenses. These histologic data extend our previously published quantitative data, which demonstrated inexorable increases in pulmonary C. neoformans CFU in uPA⁻/⁻ mice in contrast to the progressive pulmonary clearing of the pathogen in WT mice (17), by suggesting a failure of macrophage activation sufficient for C. neoformans killing.

Comparison of Ag-specific lymphocyte proliferation in WT and uPA⁻/⁻ mice
Because Mφ require stimulation by lymphokines to acquire fungicidal activity, we next turned our attention to the lymphocyte response to C. neoformans infection. Protective cell-mediated immune defenses against C. neoformans are characterized by robust T lymphocyte proliferation within regional lymph nodes. Grossly, the draining hilar and mediastinal lymph nodes from uPA⁻/⁻ mice appeared small compared with the lymph nodes from WT mice. We compared the number of cells collected from the regional nodes of uPA⁻/⁻ mice with WT mice at 10 days post-IT inoculation C. neoformans. As shown in Fig. 2A, the regional lymph nodes from uPA⁻/⁻ mice consistently had fewer cells in response to C. neoformans infection compared with WT mice (p < 0.001). These results suggested that C. neoformans-induced T cell proliferation was impaired in uPA⁻/⁻ mice. Therefore, we compared WT and uPA⁻/⁻ T lymphocyte proliferation in vitro. For these studies, WT and uPA⁻/⁻ mice were IT inoculated with C. neoformans, and the regional lymph nodes were collected 10 days later. These primed nodal cells were plated into wells and restimulated with C. neoformans in vitro. As demonstrated in Fig. 2B, there was a comparable low level of proliferation evident in both the C. neoformans-infected (primed) WT and uPA⁻/⁻ nodal cells without rechallenge in vitro. However, when the primed WT nodal
cells were rechallenged with *C. neoformans* in vitro, a robust proliferation was induced. By contrast, when rechallenged with *C. neoformans* in vitro, the primed uPA<sup>−/−</sup> nodal cells failed to increase proliferation. Thus, T lymphocyte proliferation is impaired in the absence of uPA in response to Ag. Trivial proliferation was observed when unprimed cells from either WT or uPA<sup>−/−</sup> mice were stimulated with *C. neoformans* in vitro.

**Determination of cytokine levels in BAL fluid from C. neoformans-infected WT mice compared with uPA<sup>−/−</sup> mice**

Protective host defense against *C. neoformans* depends on the generation of a T1 profile of cytokines (29). Because the T1 cytokine, IFN-γ, is known to be critical for protective host defense against *C. neoformans*, we compared the level of IFN-γ in the BAL from WT mice with that from uPA<sup>−/−</sup> mice. As shown in Fig. 3, while the levels of IFN-γ were the same in uninfected WT and uPA<sup>−/−</sup> mice (day 0), by day 7 WT mice had nearly 10 times more IFN-γ in BAL than did uPA<sup>−/−</sup> mice (*p* < 0.005), and while the IFN-γ level in BAL was low in both WT and uPA<sup>−/−</sup> mice on day 14 after infection, the WT mice had higher IFN-γ levels in BAL compared with uPA<sup>−/−</sup> thereafter to day 42.

IFN-12 is required for polarization to a T1 phenotype during immune responses. While BAL IL-12 levels were the same in uninfected WT compared with uPA<sup>−/−</sup> mice (day 0), by day 7 the BAL from WT mice had far greater IL-12 levels than uPA<sup>−/−</sup> mice (*p* < 0.0001). This striking decrease in IL-12 in uPA<sup>−/−</sup> mice was significant at all the time points studied through day 42 post-IT. Polarization to a protective T1 phenotype in response to *C. neoformans* is profoundly impaired in the absence of uPA.
Determination of the cytokine levels generated by lung inflammatory and immune cells from C. neoformans-infected WT and uPA−/− mice in vitro

The lungs of uPA−/− mice contain only about half the number of immune and inflammatory cells compared with the lungs of WT mice during the early response to pulmonary C. neoformans infection in vivo. Therefore, we questioned whether the lack of T1 cytokines at the site of the infection (BAL) in uPA−/− mice could be due to the diminished cell numbers in the lung rather than failure of polarization to a T1 profile of cytokines.

To determine whether differences in cell number were the cause of the diminished level of T1 cytokines expressed by uPA−/− mice compared with WT mice, we processed the lungs to purified single cell suspensions and then compared, in vitro, the cytokine production by equal numbers of WT and uPA−/− lymphocytes and Mφ through day 28 post-IT inoculation. There was no difference in the level of cytokines expressed by uninfected (day 0) WT compared with uninfected uPA−/− mice for any of the cytokines evaluated. Lung cells from WT mice generate a T1 profile of cytokines in response to C. neoformans challenge (Fig. 4). Even at a constant cell number, pulmonary mononuclear cells isolated from WT mice produce far more IFN-γ in vitro than do cells isolated from uPA−/− mice for all the time points tested (post-IT day 7, \( p = 0.042 \); day 14, \( p = 0.047 \); day 21, \( p = 0.049 \); day 28, \( p = 0.010 \)). Similarly, lung cells from WT mice produce far more IL-12 than do uPA−/− mice for all the time points tested (post-IT day 7, \( p = 0.001 \); day 14, \( p = 0.026 \); day 21, \( p = 0.002 \); day 28, \( p = 0.004 \)). uPA−/− mice produce higher levels of IL-5 than do WT mice over the experimental period, although this increase was statistically significant only at day 21 post-IT inoculation. Thus, the diminution of IFN-γ and IL-12 levels seen in the uPA−/− mice compared with WT mice (Figs. 3 and 4) is not due to diminished lung mononuclear cell number, nor to a global inability to elaborate cytokines, but

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Determination of the cytokine levels generated by lung inflammatory and immune cells from C. neoformans-infected WT and uPA−/− mice in vitro. Lungs were obtained from WT and uPA−/− mice at the indicated times post-IT inoculation with C. neoformans, processed to a single cell suspension, and incubated (5 × 10⁶ cells/well/ml) for 24 h. Cytokine levels were determined in conditioned media by ELISA and expressed in picograms per milliliter. Filled bars, WT mice; hatched bars, uPA−/− mice. Data are expressed as mean ± SEM; \( n = 3–7 \). A, IFN-γ, *; \( p < 0.05 \); **, \( p < 0.01 \). B, IL-12; *, \( p < 0.03 \); **, \( p < 0.005 \); ***, \( p < 0.001 \). C, IL-5; *, \( p < 0.001 \).
rather to a failure of polarization to a T1 phenotype in the absence of uPA.

**Determination of the regional lymph node cytokine profile generated by C. neoformans-infected WT and uPA^-/- mice in response to rechallenge with C. neoformans in vitro**

To further document that the low levels of T1 cytokines observed in the lungs of uPA^-/- animals infected with C. neoformans were due to an inability to develop a T1 response, and not due to an inability to recruit T1 effector lymphocytes to the lung, we evaluated cytokine production by cells obtained from regional lymph nodes. Hilar and mediastinal lymph nodes were harvested from C. neoformans-infected WT and uPA^-/- mice at various time points after IT inoculation. These primed lymph node cells were rechallenged with C. neoformans in vitro, and cytokine production was determined after 24 h.

Regional lymph node cells from WT mice generate high levels of IFN-γ in response to Ag challenge (Fig. 5). The T1 polarization in WT mice begins very early in the immune response, so that by day 7 the WT lymph node cells produce more than four times more IFN-γ in response to C. neoformans challenge in vitro than uPA^-/- nodal cells (p = 0.006). IFN-γ levels remain significantly diminished in the absence of uPA at day 28 (p = 0.0202). Interestingly, whereas IL-2 was undetectable in BAL and lung cell-conditioned media, nodal cell-conditioned media contained IL-2. Similar to IFN-γ, WT lymphocytes produced more IL-2 than did uPA^-/- lymphocytes (day 14 post-IT, p < 0.013). By contrast, lymph node cells from uPA^-/- mice generate more IL-5. Increased levels of IL-5 (a type 2 (T2) cytokine) were seen by day 7 in the lymph node-conditioned media of uPA^-/- mice when compared with WT mice (p = 0.0274). Thus, in the absence of uPA, the cytokine profile of regional lymph nodes shifts away from a protective T1 pattern of cytokines. This change in lymphocyte cytokine profile occurs early in the course of C. neoformans infection and persists throughout the development of the immune response. These data demonstrate that uPA modulates the development of central immune responses.

**FIGURE 5.** Determination of the regional lymph node cytokine profile generated by C. neoformans-infected WT and uPA^-/- mice in response to rechallenge with C. neoformans in vitro. Hilar and mediastinal lymph nodes were obtained from WT and uPA^-/- mice at the indicated times post-IT inoculation with C. neoformans, processed to a single cell suspension, and stimulated with heat-killed C. neoformans for 24 h (5 x 10^6 cells/well/ml). Cytokine levels were determined in conditioned media by ELISA and expressed in picograms per milliliter; n = 5-12. Filled bars, WT mice; hatched bars, uPA^-/- mice. Data are expressed as mean ± SEM. A, IFN-γ; *, p < 0.03; **, p < 0.01. B, IL-2; *, p < 0.02. C, IL-5; *, p < 0.03.
Reversal of impaired pulmonary response to C. neoformans in uPA−/− mice following adoptive transfer of WT immune T lymphocytes

To confirm that the immune defects in uPA−/− mice described above are attributable to a T lymphocyte deficiency, adoptive transfer experiments were done. Because pulmonary host defense against C. neoformans is dependent on the development of specific T cell-mediated immunity and the generation of a T1 cytokine response (15, 18, 19, 21, 28), we sought to determine whether the provision of WT immune T lymphocytes would reverse these immune defects in uPA−/− mice. Freshly purified WT immune T lymphocytes were injected via tail veins into recipient uPA−/− mice the day after IT inoculation.

Two weeks later, the delayed-type hypersensitivity (DTH) response to C. neoformans of WT, uPA−/−, and adoptive transfer (AT)-uPA−/− mice was compared. By way of control, footpad swelling in naive WT and naive uPA−/− mice in response to C. neoformans was the same (60 μm). WT mice developed obvious DTH reactions to C. neoformans (210 ± 10 μm), while the uPA−/− mice had nearly no response (97 ± 20 μm; WT compared with uPA−/−, p < 0.003, n = 3). By contrast, AT-uPA−/− mice developed a robust DTH response of 319 ± 45 μm (uPA−/− compared with AT-uPA−/−, p = 0.016, n = 3). Furthermore, the DTH response of the AT-uPA−/− mice was no different from the DTH response in WT mice (WT compared with AT-uPA−/−, p = NS, n = 4). Thus, the uPA−/− mice lacked the ability to respond to Ag with a DTH response, indicating impaired T cell-mediated immune function; this defect could be corrected with adoptive transfer of WT immune T lymphocytes.

Production of the T1 cytokines IFN-γ and IL-12 was compared among WT, uPA−/−, and AT-uPA−/− mice 3 wk after IT inoculation with C. neoformans. Equal numbers of purified lung immune and inflammatory cells were rechallenged with C. neoformans in vitro, and the levels of IFN-γ and IL-12 in the conditioned media were determined 24 h later. The uPA−/− cells produced far less IFN-γ than did the WT cells (372.7 ± 61 vs 2088.4 ± 459 pg/ml, p = 0.03, n = 3). In contrast, the AT-uPA−/− mice produced 1296.3 ± 167.8 pg/ml IFN-γ, far more than the uPA−/− mice (uPA−/− compared with AT-uPA−/−, p ≤ 0.009, n = 3). Furthermore, IFN-γ production by AT-uPA−/− mice was no different from IFN-γ production in WT mice (WT compared with AT-uPA−/−, p = NS, n = 5). Similarly, the uPA−/− cells produced far less IL-12 than did the WT cells (497.3 ± 7.9 vs 932 ± 58.7, p ≤ 0.002, n = 3). In contrast, the AT-uPA−/− mice produced 812.6 ± 30.3 pg/ml IL-12, far more than the uPA−/− mice (uPA−/− compared with AT-uPA−/−, p ≤ 0.0002, n = 3). Furthermore, IL-12 production by AT-uPA−/− mice was no different from IL-12 production in WT mice (WT compared with AT-uPA−/−, p = NS, n = 5).

The provision of immune WT T lymphocytes to uPA−/− mice during the immune response to C. neoformans substantially reverses the immune deficit seen in uPA−/− mice. The development of a DTH response and the generation of protective T1 cytokines in the AT-uPA−/− mice were far greater than those in the uPA−/− mice. Adoptive transfer of immune WT T lymphocytes to uPA−/− mice resulted in IFN-γ and IL-12 production that was the same as that seen in WT mice. Thus, T lymphocyte defects contribute significantly to the immune deficit noted in uPA−/− mice.

Discussion

Our study provides a mechanism for the previously reported host defense defect in uPA-deficient mice. We show that uPA−/− mice have an inability to generate a T1 response in the lung during pulmonary C. neoformans infection. The deficiency of T1 cytokines in the lung is due to 1) a decreased number of recruited effector T lymphocytes in the lungs and 2) a deficiency in T1 cytokine production by uPA−/− pulmonary mononuclear cells. Therefore, uPA−/− mice have both qualitative abnormalities in T lymphocyte function and quantitative abnormalities in T lymphocyte recruitment to sites of infection. The importance of uPA is not limited to effector cells at the site of infection. Central immune processes localized in regional nodes are markedly aberrant in the absence of uPA. Nodal T lymphocytes in C. neoformans-infected uPA−/− mice are profoundly deficient in proliferative responses, and in the production of T1 cytokines. The absence of uPA results in defects in both innate and specific immune cells. These abnormalities, present both at sites of infection and at sites of T cell differentiation (nodes), result in the lethal defect in cell-mediated immune responses seen in uPA−/− mice in response to an opportunistic fungal pathogen. uPA−/− mice have fewer pulmonary T lymphocytes in response to C. neoformans infection than WT mice (17). But most striking is the profound degree of immunocompromise in the uPA−/− mice. The uPA−/− mice respond to an opportunistic infection in a manner similar to athymic or CD4-depleted mice (14, 15). This immunocompromise is seen despite 1) an increase in CD4 cell number in uninfected uPA−/− mice compared with the WT and 2) an ~50% decrease in the number of pulmonary CD4+ cells in infected uPA−/− mice compared with WT. Because clinically overwhelming C. neoformans infection is generally not seen until patients are critically CD4 depleted (CD4 counts of <200) (30), it is difficult to attribute the degree of compromise seen in the uPA−/− mice to cell number alone, making qualitative T lymphocyte defects a more likely explanation.

Clonal expansion of lymphocytes in response to Ag is perhaps the most fundamental aspect of central immune processes. Lymphocyte proliferation in response to C. neoformans in vivo was strikingly diminished in the absence of uPA, as reflected by regional lymph node cell number during infection. Furthermore, lymphocyte proliferation in response to C. neoformans in vitro was profoundly reduced. T lymphocyte proliferative responses are deficient in uPA−/− mice. Lymphocyte proliferation in response to mitogen is diminished in uPA−/− mice compared with WT (31). The reduction seen in Ag-specific proliferation in vitro is even more striking and is paralleled in vivo by the persistently diminished number of lymph node cells seen in the uPA−/− mice throughout the 42 days of C. neoformans infection (data not shown). Thus, lymphocyte proliferative responses are profoundly blunted in the absence of uPA in an Ag-specific in vivo model.

In addition to proliferating, T lymphocytes express cytokines critical to the generation of specific immune responses and to macrophage activation. The importance of the T1 cytokine IFN-γ is well established. IFN-γ mediates Mφ activation and C. neoformans killing in vitro, and animals depleted of IFN-γ by Ab treatment are unable to defend against C. neoformans in vivo (18, 19, 32–34). It is likely that the poor intracellular killing evident in Mφ from uPA−/− mice in vivo is largely due to diminished IFN-γ stimulation. IL-2 also plays an important role. IL-2 has been shown to increase fungastic activity of human PBMCs (20), and the development of amplified anticytotoxic cDTH response in recipients of adoptively transferred spleen cells occurs when the donor cells express high levels of both IL-2 and IFN-γ (35). We show that uPA has profound regulatory effects on cytokine expression in vivo. The WT mice express a T1 profile of cytokine with high levels of IFN-γ and IL-12 in BAL fluid. Similarly, a T1 profile is clearly dominant when cell number is rigorously controlled using isolated lung cells or lymph nodes cells stimulated with C. neoformans in vitro. By contrast, the uPA−/− mice did not
polarize the immune response to a T1 profile of cytokines. The uPA−/− mice had far lower levels of IFN-γ and IL-12 in BAL fluid and in supernatants from lung cells in culture; additionally, higher levels of IL-5, a T2 cytokine, were present. Similarly, the nodal cells from uPA−/− mice produced less IFN-γ and IL-2 than did WT mice, but higher levels of IL-5. Providing WT immune T lymphocytes by adoptive transfer to C. neoformans-infected uPA−/− mice substantially reverses the immune deficits in uPA−/− mice, as demonstrated by the restoration of the DTH reaction to C. neoformans, and the generation of a T1 cytokine profile in AT-uPA−/− mice that was the same as that seen in WT mice.

These findings demonstrate that, in the absence of uPA, polarization to a protective T1 response fails to occur. This alone is sufficient to explain the death of C. neoformans-infected uPA−/− animals. Interestingly, however, the uPA−/− mice also fail to develop a well-polarized T2 response. Notably, they did not produce any detectable IL-4, nor did they have increased numbers of pulmonary eosinophils (17). WT mice of this background, inoculated with the same strain of C. neoformans, have recently been shown to generate T1 responses similar to those described in this work; however, mice of this background, when rendered genetically CCR2 deficient, generate robust T2 responses to the same pathogen (23). Thus, the uPA−/− mice are aberrant not only in their lack of a T1 response but, perhaps more importantly, in their striking unresponsiveness to Ag stimulation, and the lack of polarization to either a T1 or a T2 response.

The biologic action of uPA is traditionally conceptualized in terms of its enzymatic capacities that permit pericellular proteolysis. In vitro support for the role of uPA in cell migration has been demonstrated in hemopoietic and tumor cells, in which migration can be impeded by inhibitors to uPA or plasmin (4, 36–38). In vitro monocyte chemotaxis in the absence of matrix proteins is not diminished by inhibiting uPA enzyme activity, but is diminished by antisense-uPA oligonucleotides, suggesting that uPA binding to its receptor is involved in cell movement, perhaps by providing activation signaling (39). The binding of uPA to its receptor has been shown to activate calcium flux, prime for superoxide production, and induce tyrosine phosphorylation events (40–42). Collectively, these data argue that the biologic effects of uPA are not limited to its known ability to mediate pericellular proteolysis, but extend to cellular signaling and cell activation events.

There is substantial evidence that the uPA/uPAR system and the immune system interact. Both uPA and uPAR expression are modulated by inflammatory mediators. Mφ uPA expression is increased by TNF-α, IFN-γ, IL-1, and IL-2 (43, 44). Activated T lymphocytes express uPA and uPAR (7, 10, 11). Stimulation with phorbol esters and TCR-mediated stimulus result in substantial up-regulation of uPA and uPAR in T cells (7, 10, 11). uPAR is coexpressed with CD25 (IL-2R), establishing its expression in TCR-mediated T cell activation (7). Furthermore, uPAR is up-regulated by exposure to IL-2 and IL-4, but not to several other cytokines (7). Interestingly, exposure to IL-2 or IL-4 up-regulates uPAR on only a subset of lymphocytes, perhaps corresponding to T1 or T2 phenotypes, respectively. Conversely, there is substantial, but fragmented, evidence suggesting that uPA is a modulator of immune and inflammatory responses. For example, early reports showed that plasminogen activator could act as a lymphocyte mitogen (45), that uPA was a neutrophil chemotaxin (46), and that IL-2 synthesis by Jurkat T cells requires a membrane-associated serine protease (47). Many of these early studies were limited by an inability to characterize the involved proteases completely. uPA amplifies TNF-α production on the mRNA level by U937 Mφ (48), establishing that uPA can act as a regulator of cytokine expression in vitro. Thus, bidirectional communication links the uPA/uPAR system and inflammatory cytokine networks.

The profound effects that uPA deficiency has on T lymphocyte proliferation in response to TCR-mediated signaling, and the effects of uPA deficiency on T1/T2 differentiation during C. neoformans infection place uPA in essential T lymphocyte signaling pathways. The defect in the immune response in the absence of uPA has several characteristics. First, the defect is evident in central lymphocyte differentiation (in the regional lymph nodes); second, it is present early (by day 7) and is persistent; third, it is characterized by a profound defect in proliferation; fourth, there is diminished IL-2 production; and finally, fifth, there is a failure to polarize to a T1 phenotype. Collectively, these characteristics are remarkably similar to the responses seen when the anergic state is induced in pluriplotent precursor clones (Th0) in vitro. Anergic Th0 clones exhibit reduced proliferative responses and diminished expression of T1 cytokines with retention of T2 cytokines (49). In our in vivo model, specific T lymphocyte proliferation is nearly absent, and T1 cytokines are profoundly reduced, while IL-5 levels are minimally increased in the absence of uPA, fully consistent with anergic Th0 clones. However, whereas in vitro the separation of anergic Th0 clones and T2 clones is sometimes controversial, in vivo the uPA−/− mice clearly fail to develop a classic T2 phenotype; they remain immunologically unresponsive, despite continued Ag stimulation from proliferating microbes. This suggests that uPA deficiency may induce a functional anergy-like state in a clinically relevant, in vivo, infectious model.

In summary, uPA-replete mice develop classic T1 responses in lung and regional lymph nodes in response to C. neoformans and are protected. In the absence of uPA, T1 responses fail to develop either in the lung or in regional lymph nodes. Ag-specific lymphocyte proliferation is markedly blunted, and the uPA−/− mice fail to mount adequate immune defense against C. neoformans. These studies define uPA as a novel and required immunomodulatory molecule for the development of protective T1 immune responses to a clinically relevant fungal pathogen.

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