Interleukin-12 from Intrinsic Cells Is an Effector of Renal Injury in Crescentic Glomerulonephritis

JENNIFER R. TIMOSHANKO, A. RICHARD KITCHING, STEPHEN R. HOLDSWORTH, and PETER G. TIPPING

From the Center for Inflammatory Diseases, Monash University, Department of Medicine, Monash Medical Center, Clayton, Victoria, Australia.

Abstract. Interleukin-12 (IL-12) directs the cognate nephritogenic T helper type 1 responses that initiate renal injury in murine crescentic glomerulonephritis (GN). The recent demonstration of IL-12 production by intrinsic renal cells, including mesangial and proximal tubular cells, raises the possibility that IL-12 from nonimmune cells may contribute to inflammatory renal injury. To address this possibility, the development of sheep anti-mouse glomerular basement membrane globulin–induced crescentic GN was studied in C57BL/6 wild-type (WT), IL-12–deficient (IL-12 \(-/-\)), and IL-12 “chimeric” mice. IL-12 chimeric mice were produced by transplantation of WT bone marrow into IL-12 \(-/-\) mice to restore IL-12 production by immune cells, while leaving them deficient in renal IL-12 production. WT and “sham” chimeric mice (normal bone marrow transplanted into WT mice) developed crescentic GN with glomerular T-cell and macrophage recruitment and impaired renal function (elevated proteinuria and serum creatinine) 10 d after initiation of GN. IL-12 \(-/-\) mice showed significant protection from GN. Chimeric IL-12 mice showed significant attenuation of crescent formation, glomerular T-cell and macrophage accumulation, and renal impairment, compared with WT and sham chimeric mice, but were not protected to the same extent as IL-12 \(-/-\) mice. IL-12 chimeric mice showed no attenuation of their systemic cognate immune response to the nephritogenic antigen (sheep globulin), indicated by antigen-specific circulating antibody and cutaneous delayed-type hypersensitivity. These studies indicate that IL-12 produced by non–bone marrow derived intrinsic renal cells contributes to immune renal injury. They provide the first in vivo demonstration of a proinflammatory role for an intrinsic renal cell–derived cytokine in renal inflammation.

Glomerular crescent formation is a feature of the most severe forms of human glomerulonephritis (GN). The association of T cells and macrophages, proliferation of glomerular epithelial cells, and fibrin deposition in human (1,2) and experimental crescentic GN (3,4) suggests that this form of glomerular inflammation results from a delayed-type hypersensitivity (DTH)-like cell-mediated, cognate immune response. Glomerular injury has been demonstrated to be dependent on CD4\(^+\) T cells but not on CD8\(^+\) T cells or autologous Ig in murine crescentic GN (4,5). Studies in planted antigen and autoimmune models of crescentic GN indicate that Th1 subset responses are pivotal in directing crescentic glomerular injury. T helper type 1 (Th1)-prone strains of mice are more susceptible to crescentic injury than are Th2-prone strains (4,6). Attenuation of the primary Th1 immune response by immunological inhibition or genetic deletion of Th1 cytokines interferon-γ (IFN-γ) (4,7) and interleukin-12 (IL-12) (8) or by administration of Th2 cytokines IL-4 and IL-10 (9) attenuates crescentic GN. Augmentation of Th1 responses by administration of IL-12 (8) or genetic absence of IL-4 (10) or IL-10 (11) exacerbates crescentic GN.

IL-12 is a heterodimeric cytokine composed of two covalently linked subunits, p35 and p40, encoded by separate genes (12). Macrophages and monocytes produce IL-12 as an early response to antigenic stimuli (13). Antigen-presenting cells produce IL-12 after engagement with activated T cells and co-stimulation via CD40 ligand (14). IL-12 primes CD4\(^+\) T cells for high IFN-γ production (14,15) and polarizes uncommitted T cells toward a Th1 profile. The role of IL-12 in immune responses in vivo has been referred to as a “functional bridge” between the early noncognate innate resistance and subsequent antigen-specific adaptive immunity (13,16,17).

IL-12 has been demonstrated in crescentic glomeruli of mice that are susceptible to autoimmune anti–glomerular basement membrane (anti-GBM)-induced GN but was not detectable in strains that are resistant to the development of crescentic glomerular lesions (6). In the autoimmune GN associated with the “lupus like” syndrome of MRL/Fas\(^{lpr}\) mice, intrarenal IL-12 expression by infiltrating mononuclear cells and tubular epithelial cells was demonstrated in association with increased IFN-γ mRNA (18). In vitro, renal tubular epithelial cells produce IL-12 mRNA and low levels of IL-12 protein (18), and mesangial cells produce IL-12 mRNA and protein after lipopolysaccharide or tumor necrosis factor-α stimulation (19). Mesangial cells also express the IL-12 β1 chain receptor and...
respond directly to IL-12 stimulation by production of platelet-activating factor and reactive oxygen species (19). Therefore, in addition to the critical role for IL-12 in directing nephritogenic Th1 responses, IL-12 produced by intrinsic renal cells has the capacity to act as an effector cytokine in crescentic GN.

The current studies evaluated the capacity of IL-12 produced by intrinsic renal cells to act as a local effector molecule in Th1-dependent cell-mediated renal inflammation by studying the development of crescentic GN in “IL-12 chimeric” mice with absent intrinsic renal cell IL-12 production but normal IL-12 production from bone marrow-derived immune/inflammatory cells.

Materials and Methods

Mice

Breeding pairs of mice with targeted disruption of the IL-12 p40 gene (IL-12 −/−) (20), which have been backcrossed for nine generations onto a C57BL/6 background, were obtained from Jackson Laboratories (Bar Harbor, ME). C57BL/6 mice were used as wild-type (WT) controls, and all mice were housed and bred under specific pathogen free (SPF) conditions at Monash University (Clayton, Victoria, Australia). All animal experimentation described in this article was conducted in accordance with the Monash Medical Center Animal Ethics Committee guidelines.

Bone Marrow Transplantation

Five- to 6-wk-old male recipient IL-12 −/− or C57BL/6 mice received 1100 rads of total body irradiation. Bone marrow cells were harvested aseptically from the femora and tibiae of SPF WT donor mice, and red blood cells were lysed. Recipient mice received 5 × 10⁶ nucleated cells intravenously within 6 h of irradiation. Mice were maintained under SPF conditions for 8 wk to allow bone marrow reconstitution.

Assessment of Bone Marrow Engraftment and Lymphocyte Subset Reconstitution

Circulating leukocyte numbers and lymphocyte subsets were assessed 8 wk after bone marrow transplantation.

Circulating Leukocyte Numbers. Blood was collected into 3.3% sodium citrate, and circulating leukocyte numbers were determined by counting in a hemocytometer after lysis of red blood cells.

Circulating Lymphocyte Subsets. Citrated blood was incubated with FITC-conjugated anti-mouse CD4, anti-mouse CD8, and anti-B220 monoclonal antibodies (mAb; PharMingen, San Diego, CA), and lymphocyte subsets were quantified by flow cytometry, as described previously (21).

Lymphocyte Subset and Macrophage Repopulation in the Spleen. Splenic tissue was fixed in periodate/lysine/paraformaldehyde for 4 h, washed in 7% sucrose, frozen in liquid nitrogen, and stored at −70°C. Tissue sections (6 μm) were stained to demonstrate T cells and macrophages using a three-layer immunoperoxidase technique as described previously (3). The primary antibodies were GK1.5 (anti-mouse CD4 mAb, American Type Tissue Culture Collection [ATCC], Manassas, VA) and M 1/70 (anti-mouse Mac-1 mAb, ATCC).

Induction of GN

Anti-GBM globulin was prepared from serum of a sheep immunized against a particulate fraction of mouse GBM as described previously (22). Male WT, IL-12−/− (IL-12+/−), bone marrow transplanted WT (sham chimeric), and bone marrow transplanted IL-12 −/− (IL-12 chimeric) mice, all 13 to 14 wk of age, were sensitized by subcutaneous injection of 100 μg of sheep globulin in 100 μl of CFA. Ten d later, GN was initiated by intravenous administration of 4.4 mg sheep anti-mouse GBM globulin. The development of GN was assessed 10 d after administration of anti-GBM globulin.

Histologic Assessment of Glomerular Injury

Glomerular Crescent Formation. Kidney tissue was fixed in Bouin’s fixative and embedded in paraffin, and 3-μm sections were stained with periodic acid-Schiff (PAS) reagent. Glomeruli were considered to exhibit crescent formation when two or more layers of cells were observed in Bowman’s space. A minimum of 50 glomeruli were assessed to determine the crescent score for each animal.

Glomerular T-Cell and Macrophage Accumulation. Kidney tissue was fixed in periodate/lysine/paraformaldehyde, sectioned, and stained in an identical manner as that described for spleen to demonstrate CD4^+ T cells and macrophages. A minimum of 20 equatorial sectioned glomeruli were assessed per animal, and results were expressed as cells per glomerular cross section (c/gcs).

Tubulointerstitial Infiltration. The number of interstitial cells was counted by means of a 10-mm² graticule fitted in the eyepiece of the microscope. Five randomly selected cortical areas, which excluded glomeruli, were counted for each animal. Each high-power field represented an area of 1 mm², and counts were performed in a blinded protocol. Data are expressed as cells/mm² and represent the mean ± SEM for animals in each group.

Functional Assessment of Glomerular Injury

Mice were housed individually in cages to collect urine before administration of anti-GBM globulin and over the final 24 h of the experiment. Urinary protein concentrations were determined by a modified Bradford method (23). Serum creatinine concentrations were measured by the alkaline picric acid method using an autoanalyzer (Cobas Bio, Roche Diagnostics, Basel, Switzerland).

Histologic Demonstration of IL-12 Expression

Cryostat cut snap-frozen kidney tissue sections (6 μm) were stained for IL-12 and macrophages by direct immunofluorescence using mAb conjugated with Alexa Fluor dyes. Rat anti-mouse IL-12 mAb (anti-IL-12 p40, clone 15.6, a gift of Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA) was conjugated with Alexa Fluor 594 dye (Molecular Probes, Eugene, OR, absorption 590 nm, and fluorescence emission 671 nm equivalent to the spectra for Texas Red). Rat anti-mouse macrophage mAb M 1/70 was conjugated with Alexa Fluor 488 (Molecular Probes, absorption 494 nm, and fluorescence emission 519 nm equivalent to the spectra for FITC). Staining of tissue from IL-12−/− deficient mice provided a negative control. Sections were incubated concurrently with both antibodies at a final dilution of 1:50 for 60 min at room temperature and were examined by confocal microscopy. Confocal images were collected using a confocal inverted Nikon Diaphot 300 microscope (Bio-Rad, Hercules, CA) equipped with an air-cooled 25-mWat argon/krypton laser with lines at 488, 567, and 647 nm and triple dichroic and 560 dichroic long pass filter sets. Digital images were produced from an average of 8-line scans of approximately 1 s duration with the oil immersion lens ×40 and were collected by using a Pentium 90 computer scan control and the image acquisition and image analysis software packages COSMOS (Bio-Rad).
Assessment of the Systemic Immune Response to Sheep Globulin

Cutaneous DTH. Sensitized mice that developed GN were challenged 24 h before the end of the experiment by intradermal injection of 500 μg of sheep globulin in 50 μl of phosphate-buffered saline into the plantar surface of a hind footpad. A similar dose of an irrelevant antigen (Ag; horse globulin) was injected into the opposite footpad as a control. Footpad swelling was quantified 24 h later using a micrometer. Ag-specific DTH was taken as the difference in skin swelling between sheep globulin– and horse globulin–injected footpads and expressed as footpad thickness (mm).

Measurement of Circulating Mouse Anti-Sheep Antibody. Mouse anti-sheep globulin antibody (Ab) titers were measured by enzyme-linked immunosorbent assay on serum collected at the end of each experiment as described previously (7). Serial dilutions of serum were assayed using sheep globulin (10 μg/ml) as the capture Ag and horseradish peroxidase– conjugated sheep anti-mouse Ig Ab (Amer sham, Little Chalfont, UK) as the detecting Ab.

Statistical Analyses

Assessment of bone marrow engraftment by analysis of circulating lymphocyte subsets was performed on two separate occasions in a total of 11 IL-12 chimeric and 8 sham chimeric mice. Age-matched WT mice (n = 6) served as controls. The development of GN was studied in groups of WT, IL-12 chimeric, and sham chimeric mice on two separate occasions and on a single group of IL-12−/− mice. All mice were males between 13 and 14 wk of age. The total numbers of mice with GN in each group were as follows: WT, n = 6; IL-12 chimera, n = 8; sham chimera n = 5; and IL-12−/−, n = 4. Results are expressed as the mean ± SEM. The statistical significance was determined by one-way ANOVA, followed by Fisher’s protected least significant difference post hoc analysis.

Results

IL-12 Deficiency Protects against Development of Crescentic GN and Cutaneous DTH

Normal renal histology was unaffected by bone marrow transplantation (Figure 1, A through C). Sensitized WT mice developed proliferative GN with crescent formation and interstitial inflammatory infiltration 10 d after administration of anti-GBM globulin (Figure 1D). CD4+ cells (Figure 2A) and macrophages (Figure 2B) were observed in glomeruli. IL-12 expression was not detectable in glomeruli of normal WT mice but was expressed in glomeruli and tubules after induction of GN (Figure 2, C and D). In WT mice, glomerular IL-12 expression was observed in association with intraglomerular macrophages but also in areas where macrophages were not present. IL-12 expression did not co-localize with periglomerular macrophages (Figure 2D) or with interstitial macrophages (Figure 2C). IL-12−deficient mice showed significant protection against development of crescentic GN compared with WT mice (Figure 1F), and IL-12 was undetectable in their glomeruli (Figure 2F). The incidence of crescent formation (IL-12−/−, 7 ± 0.6% of glomeruli; WT, 32 ± 3%; P < 0.0001) and glomerular infiltration of CD4+ T cells (IL-12−/−, 0.5 ± 0.1 c/gcs; WT, 1.2 ± 0.1; P < 0.0001) and macrophages (IL-12−/−, 0.6 ± 0.1 c/gcs; WT, 2.0 ± 0.2; P < 0.0001) was significantly lower in IL-12−/− mice compared with WT mice.

Figure 1. Photomicrographs demonstrating the histologic appearances of kidneys from wild type (WT; A), interleukin-12 (IL-12) chimeric (B), and IL-12−deficient (C) mice before induction of glomerulonephritis (GN). After induction of GN, WT mice developed proliferative GN with prominent crescent formation (D). Proliferative GN was less severe and crescent formation was less frequent in IL-12 chimeric mice compared with WT mice (E), and only mild glomerular hypercellularity was apparent in IL-12−deficient mice (F). Magnifications: ×200 in A through C (periodic acid-Schiff [PAS] stain); ×400 in D through F (PAS stain).
significantly reduced (Figure 3). Reduction in the histologic appearances of renal injury and the glomerular mononuclear and interstitial cell infiltrate (IL-12$^{−/−}$, 64.5 ± 3.6 cells/mm²; WT, 141.3 ± 4.2 cells/mm²; P < 0.001) was associated with significant protection of renal function. This was indicated by reductions in proteinuria (IL-12$^{−/−}$, 1.2 ± 0.1 mg/24 h; WT, 3.9 ± 0.8 mg/24 h; P < 0.005) and serum creatinine (IL-12$^{−/−}$, 20 ± 2 μmol/L; WT, 35 ± 3 μmol/L; P < 0.005; Figure 4).

IL-12-deficient mice showed attenuation of DTH to the nephritogenic antigen as indicated by significantly reduced cutaneous swelling after an intradermal challenge with sheep globulin (IL-12$^{−/−}$, 0.28 ± 0.03 mm; WT, 0.81 ± 0.07 mm; P < 0.001). The serum titers of autologous anti-sheep globulin Ab were similar in WT- and IL-12-deficient mice (Figure 5), indicating that the humoral immune response to the nephritogenic antigen was unaffected by IL-12 deficiency.

“Chimeric” IL-12 Mice Have Normal Lymphocyte Subsets and Normal Systemic Immune Responses to Sheep Globulin

Bone marrow engraftment, studied 8 wk after transplantation, demonstrated that circulating white blood cell numbers, T-cell subsets, and B cells in IL-12 and “sham” chimeras were equivalent to those in WT mice (Table 1). Their splenic architecture with regard to distribution of CD4$^{+}$ T cells and macrophages was normal (data not shown). After cutaneous sensitization and intravenous administration of sheep globulin, IL-12 and sham chimeric mice showed identical systemic immune responses to the nephritogenic antigen as WT mice. The serum levels of mouse anti-sheep globulin Ab were identical (Figure 5, and cutaneous DTH after antigen challenge in IL-12 chimeras (Ag-specific skin swelling, 0.74 ± 0.05 mm) was equivalent to that in WT (0.81 ± 0.07 mm) and sham chimeric mice (0.66 ± 0.05 mm). This indicated functional restoration of immune competence in engrafted mice and suggests that IL-12 production by non–bone marrow-derived cells is not required for the full expression of these humoral and cell mediated systemic responses.

IL-12 Production by Intrinsic Renal Cells Is Required for Full Expression of Crescentic GN

Sham chimeric mice developed crescentic GN, with similar histologic features to WT mice with GN, indicating that bone marrow transplantation per se did not affect the development of this disease. The incidences of crescentic glomeruli (WT, 31 ± 3%; sham chimeras, 33 ± 1%) and glomerular infiltration of
CD4$^+$ T cells (WT, 1.2 ± 0.1 c/gcs; sham chimeras, 1.1 ± 0.05 c/gcs) and macrophages (WT, 3.3 ± 0.4 c/gcs; sham chimeras, 3.2 ± 0.1 c/gcs) were equivalent (Figure 3). Similarly, there was no difference in functional renal injury between the two groups, indicated by proteinuria (WT, 3.9 ± 0.8 mg/24 h; sham chimeras, 4.6 ± 0.1 mg/24 h) and serum creatinine (WT, 35 ± 3 μmol/L; sham chimeras, 36 ± 6 μmol/L). The interstitial inflammatory injury was equivalent (WT, 141 ± 4.2 cells/mm$^2$; sham chimeras, 147 ± 5.5 cells/mm$^2$; Figure 4).

However, despite equivalent systemic immune responses to the nephritogenic antigen to WT and sham chimeric mice, IL-12 chimeric mice developed significantly attenuated crescentic GN, indicating that IL-12 from intrinsic renal cells is required for full expression of immune renal injury in this model. IL-12 staining in the chimeric mice demonstrated localization of IL-12 expression on the surface of intraglomerular macrophages and limited expression in areas immediately surrounding these macrophages. Periglomerular macrophages did not express IL-12, and IL-12 expression was not detected in the tubules (Figure 2E). Histologic appearances of crescentic GN were attenuated (Figure 1E), and the incidence of crescentic glomeruli was significantly reduced (IL-12 chimeras, 16 ± 0.6%; $P < 0.0001$ compared with WT and sham chimeras). Glomerular accumulation of CD4$^+$ T cells (IL-12 chimeras, 0.7 ± 0.1 c/gcs; $P < 0.0001$) and macrophages (IL-12 chimeras, 1.7 ± 0.2 c/gcs; $P < 0.0001$) was reduced compared with both WT and sham chimeric mice with GN (Figure 3). Proteinuria was reduced in IL-12 chimeras (1.9 ± 0.4 mg/24 h; $P < 0.01$) compared with WT and sham chimeras. Serum creatinine levels in IL-12 chimeras with GN (26 ± 1 μmol/L; $P < 0.05$) were significantly reduced compared with WT sham chimeric mice with GN (Figure 4). The interstitial inflammatory infiltrate in IL-12 chimeric mice (IL-12 chimeras, 132 ± 5.0 cells/mm$^2$) was not significantly reduced compared with WT and sham chimeric mice (Figure 4).

**Discussion**

IL-12 promotes Th1 responses that are protective against intracellular pathogens such as *Leishmania*, *Listeria*, *Toxoplasma*, and *Mycobacteria* (24). In murine models of organ-specific, cell-mediated immune injury such as experimental allergic encephalomyelitis (25) and autoimmune diabetes (26),

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*Figure 3.* The incidence of crescent formation (% glomeruli affected) and glomerular infiltration of CD4$^+$ T cells and macrophages (cells/ per glomerular cross-section [c/gcs]) in normal (WT), sham chimeric, IL-12 chimeric, and IL-12–deficient (IL-12 $^{-/-}$) mice developing crescentic glomerulonephritis.

*Figure 4.* Proteinuria (mg/24 h), serum creatinine (μmol/L), and interstitial cell counts (cells/mm$^2$) in WT, sham chimeric, IL-12 chimeric, and IL-12–deficient (IL-12 $^{-/-}$) mice developing GN. The dotted line represents the mean proteinuria, creatinine, and interstitial infiltrate in normal mice.
IL-12 plays an important role by directing injurious T1 responses. A similar role for IL-12 in facilitating injurious immune responses has been demonstrated in cutaneous DTH (27). A previous study, in which an anti–IL-12 mAb was used to functionally inhibit IL-12 in vivo, demonstrated that IL-12 also promotes T1 responses and renal injury in murine crescentic GN (8). The current study provides confirmation of the important role of IL-12 in cutaneous DTH and crescentic GN by demonstrating marked protection from Ag-specific skin swelling and immune renal injury in IL-12-deficient mice.

IL-12 production by nonimmune cells has been demonstrated in a variety of organs, including the kidney (6,18) and skin (28,29). However, the contribution of IL-12 production by these nonimmune cells to organ-specific immune injury is unknown. This issue was addressed in a model of immune renal injury by demonstrating marked protection from Ag-specific skin swelling and immune renal injury in IL-12-deficient mice.

Intrinsic renal cell expression of major histocompatibility complex II was demonstrated recently to be required for renal recruitment of T cells in this model of crescentic GN (33), and simultaneous expression of IL-12 by these intrinsic cells may augment T1 activation and proinflammatory effector functions of these T cells after recruitment. IL-12 has been shown to promote T1 development, stimulate T1 cell proliferation, and induce IFN-γ production by resting and activated T cells (34–36). IFN-γ stimulates the release of monocyte chemotactic protein-1 (37) and production of proinflammatory cytokines, including IL-12 (16). IL-12-deficient mice have deficient IFN-γ production (20), and IFN-γ has been demonstrated to be an important proinflammatory mediator in crescentic GN (4,7). However, one report suggests that the absence of the IFN-γ receptor does not alter crescent formation in anti-GBM nephritis (38).

In summary, these studies demonstrate that IL-12 production by non–bone marrow–derived cells is required for the full expression of a T1-dependent model of immune renal injury but not for cutaneous DTH. They provide the first demonstration of the capacity of a cytokine derived from intrinsic renal cells to augment immune renal injury and suggest that IL-12 expression was demonstrated in glomeruli and tubules of WT and sham chimeric mice with GN. In glomeruli, IL-12 was demonstrated on the surface of and adjacent to macrophages. IL-12 expression was also observed in areas of glomeruli remote from macrophages, consistent with production by intrinsic glomerular cells. Mesangial cells have been demonstrated to express IL-12 p40 mRNA and p70 protein in response to proinflammatory stimuli, including tumor necrosis factor-α and lipopolysaccharide (19). Periglomerular and interstitial macrophages showed minimal IL-12 expression, suggesting they may have a different activation status and different functions to intraglomerular macrophages.

In IL-12 chimeric mice, IL-12 expression was restricted to the surface of macrophages and their immediate surroundings. IL-12 expression was not observed in tubules and the interstitial inflammatory infiltrate was not attenuated, indicating that this infiltrate is not dependent on tubular IL-12 production. Theses studies provide the first demonstration of an important functional role for a cytokine derived from nonimmune intrinsic renal cells in immune renal injury. GN in IL-12 chimeric mice was not reduced to the extent seen in IL-12-deficient mice, indicating that the role of local IL-12 production is complementary to that of bone marrow–derived cell IL-12 production.

IL-12 from intrinsic renal cells may act in an autocrine or a paracrine manner to amplify immune renal injury. Mesangial cells have been demonstrated recently to express the IL-12 receptor and respond to IL-12 in vitro by increasing production of inflammatory mediators, platelet activating factor, and reactive oxygen species (19), consistent with an autocrine role for mesangial cell–derived IL-12 in crescentic GN. Paracrine effects of IL-12 may be exerted on intraglomerular T cells and macrophages or endothelial cells. The potential for IL-12 to increase expression of intracellular adhesion molecule 1 or E-selectin (30) or to increase production of chemokines and RANTES has been demonstrated in other diseases (31,32).

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production by intrinsic renal cells, demonstrated in vitro and in human GN (39), may be an important modulator of renal injury.

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