CUTTING EDGE

Cutting Edge: Tumor Rejection Mediated by NKG2D Receptor-Ligand Interaction Is Dependent upon Perforin[1]

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We have investigated the primary immunity generated in vivo by MHC class I-deficient and -competent tumor cell lines that expressed the NKG2D ligand retinoic acid early inducible-1 (Rae-1) β. Rae-1β expression on class I-deficient RMA-S lymphoma cells enhanced primary NK cell-mediated tumor rejection in vivo, whereas RMA-Rae-1β tumor cells were rejected by a combination of NK cells and CD8$^+$ T cells. Rae-1β expression stimulated NK cell cytotoxicity and IFN-γ secretion in vitro, but not proliferation. Surprisingly, only NK cell perforin-mediated cytotoxicity, but not production of IFN-γ, was critical for the rejection of Rae-1β-expressing tumor cells in vivo. This distinct requirement for perforin activity contrasts with the NK cell-mediated rejection of MHC class I-deficient RMA-S tumor cells expressing other activating ligands such as CD70 and CD80. Thus, these results indicated that NKG2D acted as a natural cytotoxicity receptor to stimulate perforin-mediated elimination of ligand-expressing tumor cells. ***The Journal of Immunology*****, 2002, 169: 5377–5381.**

Natural killer cells are innate effector cells playing a critical role for the defense against certain viral infections and tumors (1, 2). Recent studies have indicated that the functions of NK cells are regulated by the integration of signals from inhibitory and activating receptors (3–8). Inhibitory receptors specific for MHC class I molecules can provide protection for target cells that express normal levels of class I molecules on their surface. Three such inhibitory receptor families have been discovered and characterized: the killer cell Ig-like receptors in human, the Ly49 lectin-like receptors in mice and the CD94/NKG2A lectin-like receptors shared by humans and mice. Although those inhibitory receptors play a key role in regulating NK cells, stimulatory receptors are believed to be necessary for the initial activation of NK cells (6). More recently, several different classes of activating NK cell receptors have been described and these are specific for either classical MHC class I molecules, nonclassical MHC class I molecules, or MHC class I-related molecules of host or pathogen origin (9–16).

Of particular interest is the lectin-like NKG2D receptor expressed on NK cells, TCR$\gamma\delta^+$ T cells, CD8$^+$TCR$\alpha\beta^+$ T cells, and activated macrophages. NKG2D is associated with the transmembrane adapter protein DAP10 and the cytoplasmic domain of DAP10 contains a YxxM motif, which binds to the p85 subunit of the phosphatidylinositol 3-kinase and activates this kinase (17). Several ligands, which bind to NKG2D, are structurally related to MHC class I molecules (8, 10, 12). In humans, the polymorphic MHC class I chain-related molecules (MIC)[3]A and MICB can be recognized by NKG2D (9, 18). Unlike conventional MHC class I, those MIC proteins display up-regulated surface expression on stressed cells and are frequently overexpressed by tumors (19). Although MIC molecules have not been found in mice, the retinoic acid early inducible-1 (Rae-1) gene products and a distantly related minor histocompatibility Ag, H60, have been reported as NKG2D ligands in mice (10, 12). Recent studies show that the ectopic expression of NKG2D ligands in several tumor cell lines resulted in the rejection of the tumor cells, even when the tumor cells expressed normal levels of MHC class I molecules (20, 21).

NK cell rejection of tumors generally involves perforin (pfp)-mediated cytotoxicity and the action of secreted IFN-γ (22–25). In this study, we have investigated the primary antitumor effector mechanisms elicited by TAP-deficient RMA-S and parental RMA lymphomas expressing Rae-1β. Although Rae-1β ligation of NKG2D in vitro triggered NK cell pfp-mediated cytotoxicity and IFN-γ secretion, primary rejection of Rae-1β expressing RMA-S cells was mediated by NK cells and pfp played a dominant role in this process.

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[3] Abbreviations used in this paper: MIC, MHC class I chain-related molecule; AGM1, asialo-GM1; pfp, perforin; Rae-1, retinoic acid early inducible-1; WT, wild type.

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Materials and Methods

Mice

Inbred wild-type (WT) C57BL/6 mice were purchased from the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The following gene-targeted mice were bred at the Peter MacCallum Cancer Institute (East Melbourne, Victoria, Australia): C57BL/6 IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice, C57BL/6 pfp-deficient (pfp$^{-/-}$) mice, and C57BL/6 RAG-1-deficient (RAG-1$^{-/-}$) mice. C57BL/6 mice doubly deficient for pfp and IFN-γ (pfp$^{-/-}$IFN-$\gamma^{-/-}$) or pfp and RAG-1 (RAG-1$^{-/-}$pfp$^{-/-}$) were produced and bred at the Peter MacCallum Cancer Institute. Mice that were 6–12 wk of age were used in all experiments that were performed according to animal experimental ethics committee guidelines.

Abs and reagents

Purified mAbs reactive with mouse CD16/32 (2.4G2), mouse NK1.1 (PK136), mouse CD8 (53-6.7), and mouse IFN-γ (R4-6A2) were all purified from hybridomas. Isotype control for rat IgG1 (R3-34), rat IgG2a (R35-95) (BD PharMingen, San Diego, CA), and PE-labeled goat anti-rat IgG (Caltag Laboratories, Burlingame, CA) were purchased. Rabbit anti-asialo-GM1 (AGM1) Ab was purchased from WAKO (Richmond, VA). Human IL-2 was kindly provided by Chiron (Emeryville, CA).

Tumor cell lines

The RMA and RMA-S cell lines used in this study were derived from C57BL/6 (B6, H-2^b) mice. RMA and RMA-S are T cell lymphomas derived from the Rauscher murine leukemia virus-induced RBL-5 cell line (26). RMA-S and infectants were grown in complete RPMI 1640 medium as previously described (27). RMA-S-m (mock vector alone transfected), RMA-S-CD70, and RMA-S-Rae-1β infectants were prepared and selected by flow cytometry as previously described (27). RMA and RMA-Rae-1β tumor cells were prepared as described elsewhere (20) and analyzed by flow cytometry using PE-labeled mNKG2D tetramer as described previously (10).

NK cell culture and assay in vitro

Spleen NK cells from RAG-1$^{-/-}$ or RAG-1$^{-/-}$pfp$^{-/-}$ mice were prepared and characterized as described elsewhere (27). In coculture experiments, purified NK cells (3×10^5/well) were cocultured with irradiated (15,000 rad) RMA-S-mock, RMA-S-Rae-1β cells, or RMA-S-CD70 at indicated a responder:stimulator ratio in 96-well round-bottom culture plate. For the proliferation assay, the NK cell cultures were incubated for 2 days in the presence of IL-2 (25 U/ml). Cell-free supernatants from NK cultures were harvested after 24-h incubation and subjected to IFN-γ ELISA with a specific ELISA kit (BD PharMingen). Cytotoxic activity of NK cells was assessed as described previously (27). The anti-IFN-γ mAb (10 μg/ml) was added to the cultures for blocking IFN-γ. Each experiment was performed three times using triplicate samples.

Tumor growth assays

Groups of 5–10 untreated (WT, RAG-1$^{-/-}$, IFN-$\gamma^{-/-}$, pfp$^{-/-}$, pfp$^{-/-}$ IFN-$\gamma^{-/-}$) or Ab-treated WT (anti-AGM1, anti-NK1.1, anti-CD8) mice were injected s.c. with vector alone-infected- or Rae-1β-infected tumor cells (range, 5×10^4–5×10^6 cells) in 0.2 ml of PBS as indicated. Mice were observed every 2 days for tumor growth using a caliper square measuring along the perpendicular axes of the tumors (the product of two diameters $\pm$ SE) and sacrificed when tumors reached a size >12 mm in diameter. Mice without any signs of tumor growth were kept under observation for at least 100 days. In some experiments in vivo, to deplete NK cells, mice were treated on days -1, 0, and 7 (where day 0 is the day of primary tumor inoculation) with anti-AGM1 (200 μg i.p) or anti-NK1.1 mAb (200 μg i.p.) as described elsewhere (27). Alternatively, some groups of mice were treated as described above with anti-CD8 mAb (200 μg i.p) to deplete CD8$^+$ cells or 200 μg of control Ig.

Statistical analysis

Data were analyzed using the Mann-Whitney U test. All $p < 0.05$ were considered to be significant.

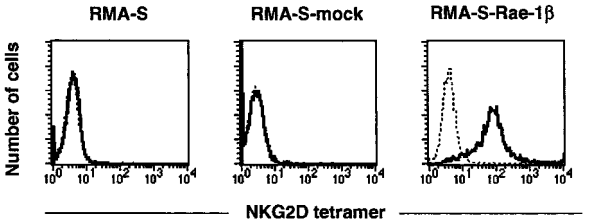

FIGURE 1. Staining of RMA-S cells and RMA-S-Rae-1β infectants with NKG2D tetramer. RMA-S and RMA-S infectants were analyzed by flow cytometry for their expression of ligands for mouse NKG2D. Cell lines were stained with tetrameric NKG2D (bold line) or with control T22-tetramer (dotted line).

Results and Discussion

Rae-1 expression induces primary tumor rejection and requires NK cells

RMA-S, a MHC class I-deficient variant of RBL-5, is one of the prototypic NK cell-sensitive target cells for in vitro and in vivo studies (22, 23, 26). To examine the role of NKG2D in NK cell-mediated tumor rejection, we prepared by retroviral infection, RMA-S cells that expressed the vector alone ($-$mock) or the NKG2D ligand Rae-1β (Fig. 1). The control and Rae-1β-expressing cell lines had similar growth kinetics in vitro (data not shown). Tumor cells that have lost MHC class I expression, such as RMA-S, are efficiently controlled in vivo by NK cells (26). Over a considerable dose range (5×10^4–5×10^6 cells), RMA-S-Rae-1β tumor cells were rejected more avidly than RMA-S-mock tumor cells (Fig. 2, *A–F*). Tumor rejection was mediated entirely

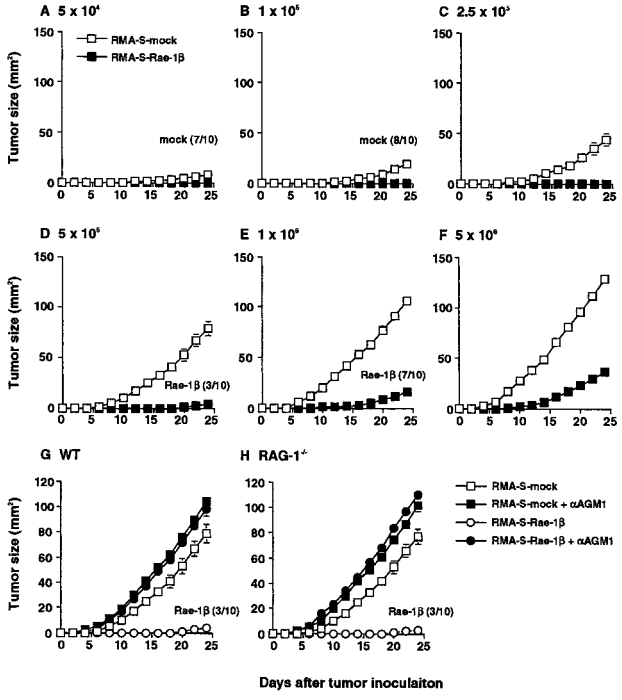

FIGURE 2. Rae-1β-expressing tumor cells are rejected by NK cells. WT mice were inoculated s.c. with RMA-S-mock or RMA-S-Rae-1β tumor cells at the indicated doses (*A–F*). Some groups of WT and RAG-1$^{-/-}$ mice were treated with anti-AGM1 and inoculated s.c. with either RMA-S-mock or RMA-S-Rae-1β (5×10^5) tumor cells (*G* and *H*). Fractions are indicated for instances where tumors grew in some, but not all, mice. Tumor growth was examined every second day and recorded data are the means $\pm$ SE of 5–10 mice in each group.

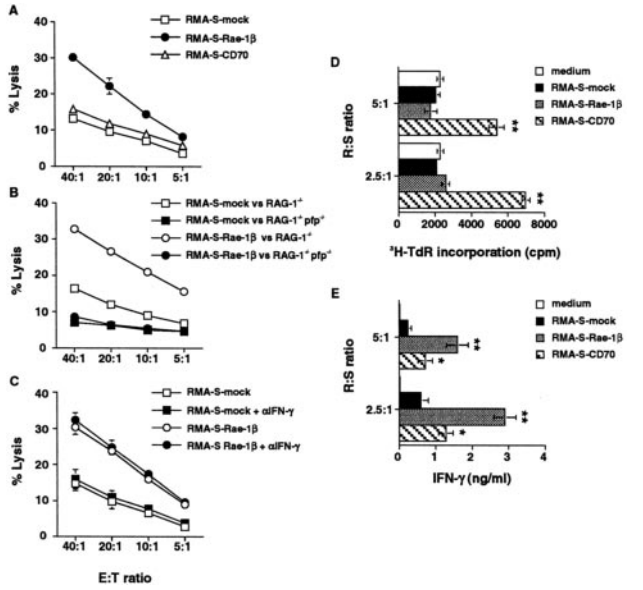

FIGURE 3. Rae-1β-expressing tumor cells trigger activation of naive NK cells. The effect of NKG2D engagement on NK cell cytotoxicity (*A–C*), proliferation (*D*), and IFN-γ production (*E*) was tested. The cytotoxicity of freshly isolated splenic NK cells from RAG-1$^{-/-}$ (*A–C*) or RAG-1$^{-/-}$pfp$^{-/-}$ (*B*) was tested against ^{51}Cr-labeled RMA-S-mock, RMA-S-Rae-1β, or RMA-S-CD70 cells (*A*) in the presence of anti-IFN-γ mAb (*C*) at the indicated E:T ratios. Freshly isolated RAG-1$^{-/-}$ splenic NK cells were cocultured with irradiated RMA-S-mock, RMA-S-Rae-1β, or RMA-S-CD70 cells. After 2 days, proliferative responses were assessed by pulsing cultures with [^{3}H]thymidine for the last 16 h (*D*). Cell-free culture supernatants were collected after 24 h and IFN-γ was measured by specific ELISA (*E*). Data are the means $\pm$ SD of triplicate wells from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ as compared with RMA-S-mock group.

by NK cells in both cases as evidenced by the increased tumor take and growth rate in WT or RAG-1$^{-/-}$ mice treated with anti-AGM1 Ab (Fig. 2, *G* and *H*). These data indicated that Rae-1β expression could enhance primary NK cell-mediated tumor rejection independently of T, NKT, or B cells.

Expression of Rae-1 triggers NK cell cytotoxicity and IFN-γ secretion, but not proliferation

We next examined whether Rae-1β expression on RMA-S cells could induce cytotoxicity (Fig. 3*A*), proliferation (Fig. 3*D*), and IFN-γ production (Fig. 3*E*) by freshly isolated naive NK cells in vitro. Rae-1β expression on RMA-S cells triggered target cell killing by NK cells (Fig. 3*A*) without prior activation and it was totally mediated by the pfp pathway (Fig. 3*B*). Although increased IFN-γ production of NK cells was observed following interaction with RMA-S-Rae-1β (Fig. 3*E*), IFN-γ did not contribute to Rae-1β-triggered cytotoxicity (Fig. 3*C*). Interestingly, Rae-1β did not induce NK cell proliferation, whereas it enhanced other defined NK cell functions, such as cytotoxicity and IFN-γ production (Fig. 3*D*). By comparison, CD70 expression on RMA-S tumor cells has been demonstrated to enhance NK cell-mediated proliferation and IFN-γ secretion, but not cytotoxicity (Fig. 3, *A*, *D*, and *E* and Ref. 27). Therefore, RMA-S-Rae-1β cells induce both cytotoxicity and IFN-γ production by NK cells in vitro.

pfp-mediated death is sufficient for Rae-1 expressing tumor rejection

Finally, we investigated the relative contribution of effector molecules in the rejection of Rae-1β-expressing primary tumors by NK cells in vivo. Fig. 4*A* shows that NK cell-mediated rejection of s.c. RMA-S tumor cells was pfp and IFN-γ dependent as previously observed (27). As previously demonstrated, CD70-expressing RMA-S cells were rejected through both pfp- and IFN-γ

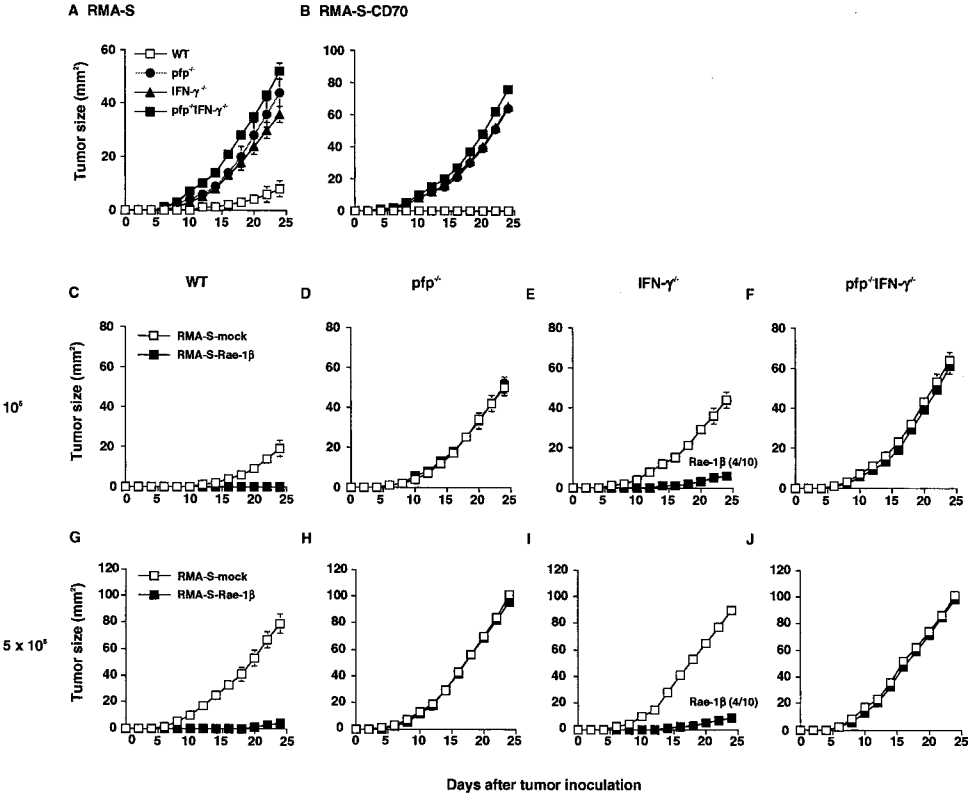

FIGURE 4. pfp-mediated cytotoxicity is required for rejection of Rae-1β-expressing tumors. Subcutaneous inoculation of RMA-S (*A*, 5×10^4) or RMA-S-CD70 (*B*, 5×10^5) cells into WT, pfp$^{-/-}$, IFN-$\gamma^{-/-}$, and pfp$^{-/-}$IFN-$\gamma^{-/-}$ mice. RMA-S-mock and RMA-S-Rae-1β (*C–F*, 10^5; *G–J*, 5×10^5) cells were s.c. inoculated into WT (*C* and *G*), pfp$^{-/-}$ (*D* and *H*), IFN-$\gamma^{-/-}$ (*E* and *I*), and pfp$^{-/-}$IFN-$\gamma^{-/-}$ mice (*F* and *J*). Fractions are indicated for instances where tumors grew in some, but not all, mice. Tumor growth was examined every second day and data recorded are the means $\pm$ SE of 5–10 mice in each group.

dependent mechanisms (Fig. 4*B* and Ref. 27). Interestingly, NK cell-mediated rejection of RMA-S-Rae-1β tumors occurred in WT (Fig. 4, *C* and *G*) and IFN-$\gamma^{-/-}$ (Fig. 4, *E* and *I*) mice, but was not observed in either pfp$^{-/-}$ mice (Fig. 4, *D* and *H*) or pfp$^{-/-}$IFN-$\gamma^{-/-}$ mice (Fig. 4, *F* and *J*). Similar results were obtained at a higher dose of RMA-S-Rae-1β tumor cells (10^6) where 7 of 10 WT mice and 4 of 5 IFN-$\gamma^{-/-}$ mice had developed tumors by day 20 (data not shown). These results indicated a critical requirement for pfp-mediated cytotoxicity in NK cell rejection of Rae-1β-expressing tumors, whereas increased IFN-γ production of NK cells (observed in vitro (Fig. 2*E*)) played little role in tumor rejection in vivo. We also observed that MHC class I-positive RMA-Rae-1β tumors cells were rejected by either NK cells or CD8$^+$ T cells at low tumor doses (data not shown and as previously described in Ref. 20). Again, rejection occurred in WT and IFN-$\gamma^{-/-}$ mice, but was not observed in pfp$^{-/-}$ or pfp$^{-/-}$IFN-$\gamma^{-/-}$ mice (data not shown). These observations were also made in NK cell-depleted or CD8$^+$ cell-depleted mice, but not in NK and CD8$^+$ cell-depleted mice. This confirmed the finding that CD8$^+$ T cells participate in the rejection of RMA-Rae-1β tumor cells (20), in contrast to another report that only NK cells mediate rejection of RMA cells transduced with Rae-1γ (21). In addition, NKG2D-mediated tumor rejection by both NK cells and CD8$^+$ T cells was pfp dependent, suggesting that in these lymphocytes NKG2D acts predominantly as a natural cytotoxicity receptor.

Potential role of NKG2D in NK cell-mediated tumor surveillance

Although several NK cell receptors have been implicated in NK cell cytolysis and tumor rejection, NKG2D was proposed to play an important role in tumor immune surveillance (8, 28) since the defined ligands are frequently overexpressed on many different tumor cells (10, 19). Our results indicated that NKG2D ligand-expressing tumor cells rapidly induced NK cell cytotoxicity in vitro without any prior stimulation and rejected tumors through a pfp-dependent mechanism in vivo. Surprisingly, IFN-γ production was not critical for NKG2D ligand-expressing tumor cell rejection despite its clear requirement in tumor rejection and the development of T cell memory induced by other NK cell-activating molecules, such as CD27 and CD28 (27, 29). It remains possible, but untested, that NKG2D fails to stimulate NK cell IFN-γ production in vivo. Alternatively, CD27 does not induce NK cell cytotoxicity, and enhanced tumor rejection triggered via CD27 or CD28 might require additional IFN-γ-dependent signals from the tumor or dendritic cells. Therefore, NKG2D may serve as a primary recognition receptor to allow elimination of "dangerous" cells, such as transformed cancer cells, stressed, or virus-infected cells, via direct pfp-mediated cytotoxicity. In particular, this would be a more rapid and powerful stimulus designed to specifically kill stressed or infected tissue cells that do not typically express ligands for other immune-activating molecules such as CD27 (CD70) and CD28 (CD80/86).

Whether or not NKG2D ligands play a critical role in immune detection of spontaneous tumor formation is as yet untested. It was suggested that NKG2D-stress ligand pathway played an important role in the regulation of carcinogen-induced cutaneous malignancy by $\gamma\delta^+$ T cells (30); however, 12-*O*-tetradecanoylphorbol-13-acetate plus 7,12-dimethylbenz(*a*)anthracene induction of skin papillomas was clearly pfp independent (31). Therefore, NKG2D recognition is not involved or another key effector pathway is triggered in $\gamma\delta^+$ T cells by NKG2D ligation. Macrophages can express NKG2D (10), but not pfp, and hence this receptor must regulate other important effector functions in vivo. Perhaps it is surprising that isolated human and rodent tumors do often express NKG2D ligands, given the powerful immunoselection pressure of pfp (32) and the potential importance of the NKG2D-stress ligand pathway in vivo (20, 21). Viruses that are sensitive to pfp and infect dendritic cells (CMV) have evolved very sophisticated means to avoid triggering the NKG2D-stress ligand pathway (33) and therefore tumor cells that express NKG2D ligands must evade elimination by either providing balancing inhibitory signals to immune effector cells or developing pfp resistance. Resistance to pfp may be a very important tumor escape mechanism to consider for future study, since should NK cells select tumor cells that are pfp resistant, adaptive immune responses using this pathway (e.g., CTL) might also be quite ineffectual. Future studies that define the unique features of NKG2D activation and elucidate the importance of other effector molecules and chemokines downstream will be of great interest.

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