T Cell–mediated Eradication of Murine Metastatic Melanoma Induced by Targeted Interleukin 2 Therapy

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Summary

Induction of a T–cell mediated antitumor response is the ultimate goal for tumor immunotherapy. We demonstrate here that antibody-targeted IL2 therapy is effective against established pulmonary and hepatic melanoma metastases in a syngeneic murine tumor model. The effector mechanisms involved in this tumor eradication are not dependent on NK cells, since the therapeutic effect of antibody-IL2 fusion protein was not altered in NK cell-deficient mice. In contrast, T cells are essential for the observed antitumor effect, since therapy with antibody-IL2 fusion proteins is unable to induce tumor eradication in T cell–deficient SCID mice. In vivo depletion studies characterized the essential effector cell population further as CD8+ T cells. Such CD8+ T cells, isolated from tumor bearing mice after antibody-directed IL2 therapy, exerted a MHC class I–restricted cytotoxicity against the same tumor in vitro. These data demonstrate the ability of antibody-targeted IL2 delivery to induce a T cell–dependent host immune response that is capable of eradicating established melanoma metastases in clinically relevant organs.

M ost progressively growing neoplasms, e.g., melanoma, do not provoke antitumor immune responses that are capable of controlling the growth of malignant cells, in spite of the fact that these cells express tumor-associated, T cell epitopes. Almost two decades ago, Talmage et al. (1) first described a tumor cell line that failed to induce an allogeneic T cell response despite its apparently normal expression of MHC molecules. Furthermore, they demonstrated that this defective T cell activation could be restored by cytokines (1); thereby providing the rationale for cytokine based forms of immunotherapy.

A therapeutic approach currently receiving much attention is the ex vivo genetic modification of tumor cells to express various cytokines (2, 3). When produced by tumors, many of these cytokines induce a local inflammatory response which results in elimination of the injected tumor cells. In some cases, systemic immune responses are generated against challenge with the wild-type parental tumor. Since most cytokines are paracrine factors working physiologically at high concentrations within a few cell diameters from their cell of origin, this gene transfer approach, in general can not be replaced by systemic cytokine administration unless the cytokine is directed preferentially to the tumor site (4).

We recently demonstrated the feasibility of an alternative therapeutic approach for cancer that combines high local concentrations of cytokines in the tumor microenviron-

ment, low systemic toxicities, and a technically simple modus operandi (5, 6). This goal was achieved by the construction of fusion proteins consisting of tumor-specific monoclonal antibodies and cytokines; thereby, employing the unique targeting ability of antibodies to direct cytokines to the tumor site. Here, we demonstrate the effectiveness of antibody-targeted IL2 therapy for established pulmonary and hepatic melanoma metastases in a syngeneic tumor model that enabled the analysis of effector mechanisms responsible for the therapy-induced tumor eradication. This analysis revealed CD8+ T cells as the essential effector population.

Materials and Methods

Cell Lines, Animals, and Reagents. The murine melanoma cell lines, B16 and B78-D14, have been described previously (7). B78-D14 was derived from B16 melanoma cells by transfection with genes coding for β-1,4-*N*-acetylgalactosaminyltransferase and α-2,8-sialyltransferase inducing a constitutive expression of the gangliosides G_{D2} and G_{D3}.

Mouse/human chimeric antibodies directed against the EGF receptor (ch225) or G_{D2} (ch14.18) were constructed by joining the cDNA for the variable region of the murine antibodies with the constant regions of the $\gamma1$ heavy chain and the κ light chain. The antibody-interleukin 2 fusion protein ch14.18-IL2 was constructed by fusion of a synthetic sequence coding for human IL2 to the carboxyl end of the human C$\gamma1$ gene followed by inser-

tion into the eukaryotic expression vector pdHL2. The resulting vector was introduced into Sp2/0-Ag14 cells. Each µg of fusion protein corresponds to approximately 3,000 IU of IL-2 activity. (5).

C57BL/6J, C57BL/6J bg/bg and C57BL/6J scid/scid mice were obtained from Jackson Laboratory at the age of 4–6 wk and housed under specific pathogen-free conditions.

In Vivo Depletion with mAb. Rat IgG_{2a} anti-CD4 (clone H129.19) and rat IgG_{2a} anti-CD8 (clone 53-6.7) mAb were used for in vivo depletion of T cell subsets (8). Protocols yielding maximum depletion of T cell subsets, i.e., over 95% as determined by indirect immunofluorescence staining and cytofluometric analysis of lymph nodes and spleens, consisted of weekly i.p. injections of 500 µg of the respective mAb.

Experimental Lung Metastases. Single cell suspension of 5 × 10^6 tumor cells in 500 µl PBS were injected into the lateral tail vein. After 7 d micrometastases were present disseminated throughout the lungs and were invading into the pulmonary alveoli. At d 28 after tumor cell injection, grossly visible metastases were present on the surface of the organ.

Experimental Hepatic Metastases. Tumor cells (2.5 × 10^6) were injected in 100 µl RPMI 1640 with a 27-gauge needle beneath the splenic capsule over a period of 60 s, followed by ligation of the splenic pedicle with a 4.0 silk suture and the removal of the spleen. 35 d after this procedure the animals were sacrificed and examined for metastases.

Biodistribution. The ch14.18-IL2 fusion protein was labeled with ^{125}I as described (6). Experimental hepatic or pulmonary metastases were induced as described above. 10 d after tumor cell inoculation, animals received one i.v. injection of 5 mCi ^{125}I-labeled ch14.18-IL2 fusion protein. Animals were killed 12 h after injection.

Statistical Analysis. The statistical significance of differential findings between experimental groups of animals was determined by student's *t* test. The nonparametric Wilcoxon rank sum test was chosen when the data were not amenable for parametric tests as defined by the David-Pearson-Stephen's test. Findings were regarded as significant if two-tailed *P* values were ≤0.01.

Results

Therapeutic Efficacy of Antibody-IL2 Fusion Proteins. We previously demonstrated that the genetic fusion of IL2 to the carboxy-terminal end of an antibody heavy chain changes neither the biological activity of IL-2 nor the binding affinity of the monoclonal antibody (5, 6). To test the effect of these antibody-IL2 fusion proteins on melanoma metastases in vivo, we employed a number of different experimental tumor models using B16 melanoma cells, which had been transfected with genes coding for β-1,4-*N*-acetylgalactosaminyltransferase and α-2,8-sialyltransferase resulting in a constitutive expression of the gangliosides G_{D2}, the antigen recognized by ch14.18-IL2. These tumor cells form experimental pulmonary and hepatic metastases following intravenous or intrasplenic injection, respectively. After 1 wk these were present as disseminated, established micrometastases.

The first series of experiments addressed the effect of the antibody-IL2 fusion protein on disseminated, established pulmonary metastases. Treatment of mice 1 wk after tumor cell inoculation by i.v. administration of 8 µg ch14.18-IL2

fusion protein for 7 d completely eradicated pulmonary micrometastases in 14 of 16 animals (Table 1). This complete eradication of micrometastases was confirmed by histologic examination of serial sections of lung specimens (data not shown). For the remaining two animals the tumor load was dramatically reduced to one or two grossly visible pulmonary foci, as compared to control animals which suffered from more than 500 pulmonary metastases, after receiving either no treatment or the combination of equimolar amounts of recombinant IL2 (24,000 I.U.) and ch14.18 mAb (8 µg). Similar results were obtained when animals bearing disseminated hepatic metastases established for 7 d were treated i.v. with 8 µg ch14.18-IL2 fusion protein for 7 d (Table 1). This treatment resulted in complete regression of micrometastases in 87.5% of these animals. The specificity of the antitumor effect of ch14.18-IL2 fusion proteins was established, since a fusion protein that is not reactive with the tumor cells completely failed to exert any antitumor effects (Table 1). Differences in the number of metastatic pulmonary foci or the hepatic metastatic score between animals receiving the specific fusion protein and those subjected to other treatments were statistically significant (*P* ≤0.002).

Although no metastatic foci could be detected by macroscopic and/or histologic examination in the animals following treatment with ch14.18-IL2 these mice could still suffer from minimal residual disease. Survival studies were performed to measure the extent of such a hypothetical residual disease. The mean survival time of mice after induction of experimental pulmonary metastases without further treatment was 41 d. This survival time was not significantly altered by the administration of ch14.18 antibody in combination with recombinant IL2 (mean = 44 d), but was more than doubled by treatment with the ch14.18-IL2 fusion protein. The same was true for mice suffering from hepatic metastases. These mice lived at least twice as long after therapy with ch14.18-IL2, than control animals (mean 51 or 55 d, respectively). Only one of the treated animals died within the observation period. At the end of the observation period a post mortem examination revealed no metastatic disease in any of the major peripheral organs or the central nervous system.

Biodistribution of ch14.18-IL2. We previously demonstrated that fusion proteins localize in antigen-expressing subcutaneous tumors in nude mice (6). However, the clinically more relevant question is whether the fusion protein is able to target micrometastases in affected organs of a syngeneic host. The biodistribution of ^{125}I-ch14.18-IL2 fusion protein in C57BL/6J mice suffering from either hepatic or pulmonary metastases, 10 d after experimental induction with B78-D14 melanoma cells, is shown in Fig. 1. The amount of radioactivity in livers and lungs of these or naive animals was determined 12 h after i.v. injection of ^{125}I-ch14.18-IL2. This analysis revealed a strong localization of the tumor specific fusion protein within the tumor bearing organs. Since only parts of the organs' tissue is involved in the metastatic process, the ratio of ^{125}I-ch14.18-IL2 fusion protein localizing to the tumor or to normal tissue can be ex-

Table 1. *Effect of the Tumor Specific Antibody-IL2 Fusion Protein on Established Pulmonary and Hepatic Metastases*

Pulmonary metastasis	Treatment*	No. of Foci
	None	254, >500, >500, >500, >500, >500, >500, >500[‡§]
		>500, >500, >500, >500, >500, >500, >500
	rIL2 + ch14.18	96, 167, 231, >500, >500, >500, >500, >500
		82, 151, 154, 163, >500, >500, >500, >500
	ch225-IL2	113, 136, 200, >500, >500, >500, >500
	ch14.18-IL2	0, 0, 0, 0, 0, 0, 1, 2
		0, 0, 0, 0, 0, 0, 0, 0
Hepatic Metastasis	Treatment	Metastatic Score[∥]
	None	2, 3, 3, 3, 3, 3, 3[§]
	rIL2 + ch14.18	2, 2, 2, 3, 3, 3, 3, 3
	ch14.18-IL2	0, 0, 0, 0, 0, 0, 0, 1

*Experimental pulmonary and hepatic metastases were induced by i.v. injection of 5 × 10^{6} or intrasplenic injection of 2.5 × 10^{6} B78-D14 cells, respectively. Treatment was started 1 wk thereafter and consisted of daily i.v. administration of PBS, 8 μg chimeric monoclonal antibody ch14.18 and 24000 IU recombinant IL2, or 8 mg of either the non-specific fusion protein ch225-IL2 or the tumor-specific fusion protein ch14.18-IL2 as indicated for seven consecutive days.

[‡]All experimental groups were started with eight mice; animals found dead before the planned killing date were not included in the evaluation.

[§]Differences in numbers of metastatic foci and scores between the fusion protein group and all control groups were statistically significant ($P \leqslant 0.001$).

[∥]Results are given as metastatic score with 0 = no visible metastatic foci, 1 = less than 5% of the liver surface covered with metastatic foci, 2 = between 5 and 50% of the liver surface covered with metastatic foci, and 3 = more than 50% of the liver surface covered with metastatic foci.

pected to be even higher than the experimental results indicate.

T Cell Dependency of Fusion Protein-induced Tumor Rejection. To test whether NK cells are essential for the antitu-
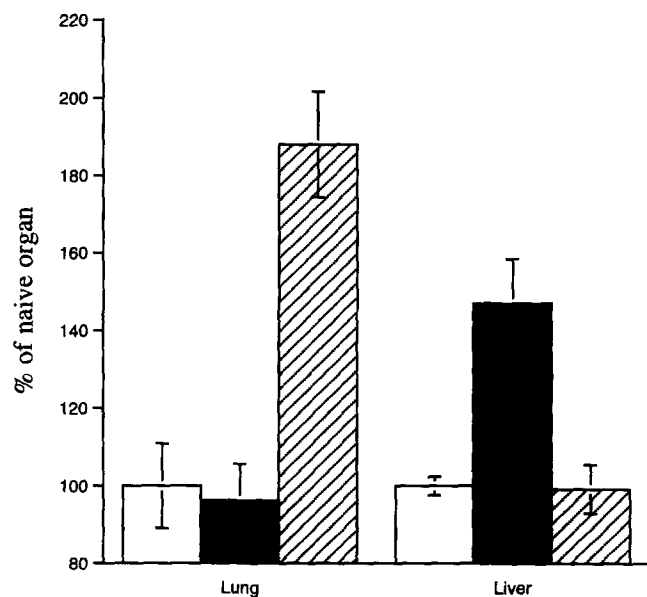

Figure 1. Biodistribution of ch14.18-IL2. C57BL/6 mice bearing either no (*white*), pulmonary (*striped*), or hepatic (*black*) metastases established for 14 d were injected with 5 μCi ^{125}I-labeled ch14.18-IL2 and the amount of radioactivity in lung or liver was measured 12 h thereafter. Each value represents the mean and standard deviation for three animals.

mor effect of antibody-IL2 fusion proteins, we induced experimental pulmonary metastases of B78-D14 cells in C57BL/6 *beige/beige* mice. Since these mice lack functional NK activity, any therapeutic host response induced is independent of NK cells. This was the case, as treatment of tumor bearing NK-deficient mice with ch14.18-IL2 caused a complete eradication of tumor micrometastases (Table 2). The second series of studies with immunodeficient mice addressed the relevance of T cells for the observed tumor eradication. B78-D14 cells were injected i.v. into T cell–deficient C57BL/6 *scid/scid* mice and therapy with ch14.18-IL2 was started 7 d thereafter. In the absence of T cells the therapeutic efficacy of the antibody-IL2 fusion protein is dramatically reduced. In fact, a complete eradication of micrometastases was not achieved in any of the animals (Table 2). This observation strongly suggests a T cell–dependent mechanism for the demonstrated antitumor effect. Thus, the participation of T cell subsets in the fusion protein-induced tumor regression was investigated by systemic in vivo depletion of T cell subpopulations with specific antibodies. Since NK cells might be able to substitute for some functions of depleted T cell populations, these studies were done in C57BL/6 *beige/beige* mice. These studies revealed that depletion of CD4+ T cells did not significantly interfere with the antibody-IL2 induced immune response (Table 2). In contrast, depletion of CD8+ or both CD4+ and CD8+ cells abrogates the therapeutic effect of the administered fusion protein. Provided that depletion of each T cell subset was effective and specific, these results indicate that

Table 2. *Effect of Antibody-IL2 Fusion Protein Therapy on Micrometastases in Immunodeficient and T Cell–depleted Mice*

Mice*	In vivo depletion‡	Treatment	No. of Foci
C57BL/6 *beige/beige*	None	None	>500, >500, >500, >500, >500, >500, >500§
	None	rIL2 + ch14.18	149, 187, >500, >500, >500, >500, >500, >500
	None	ch14.18-IL2	0, 0, 0, 0, 0, 0, 3, 21
C57BL/6 *scid/scid*	None	None	>500, >500, >500, >500, >500, >500
	None	rIL2 + ch14.18	149, 187, >500, >500, >500, >500, >500, >500
	None	ch14.18-IL2	98, 131, 150, 171, 180, 180, 200, >500
C57BL/6 *beige/beige*	None	None	>500, >500, >500, >500
	None	ch14.18-IL2	0, 0, 0, 2
	CD4	ch14.18-IL2	0, 0, 22, 62
	CD8	ch14.18-IL2	56, 79, 126, >500
	CD4 + CD8	ch14.18-IL2	78, 142, >500, >500

*Experimental pulmonary metastases were induced in C57BL/6 *beige/beige* or *scid/scid* mice by i.v. injection of 5 × 10⁶ B78-D14 cells; treatment was started 1 wk thereafter and consisted of daily i.v. administration of PBS, 8 μg chimeric monoclonal antibody ch14.18 and 24,000 IU recombinant IL2 or 8 μg of the tumor-specific fusion protein ch14.18-IL2 as indicated for 7 consecutive days.

‡C57BL/6 *beige/beige* mice were depleted of CD4+, CD8+ or both cell populations by weekly i.p. injections of 500 μg anti-CD4, anti-CD8 or both mAb starting 3 d before induction of tumors.

§All experimental groups were started with eight mice; animals found dead before the planned killing date were not included in the evaluation.

only the presence of CD8+ T cells is mandatory for tumor eradication.

The third line of evidence indicating the involvement of CD8+ T cells was provided by cytotoxicity studies. Spleen cells isolated from mice after induction of pulmonary metastases and subsequent treatment with ch14.18-IL2 displayed a high cytolytic activity against B78-D14 cells in a 4-h ⁵¹Cr-release assay (Fig. 2 *A*). Enrichment for CD8+ T cells, demonstrated that this cell population provides the

major contribution to the detected cytolytic activity (Fig. 2 *B*). Furthermore, blocking studies with antibodies against H-2Kᵇ/H-2Dᵇ antigens (clone 28-8-6, C3H IgG₂ₐ, κ) proved that the killing of B78-D14 cells by either unselected primed lymphocytes or the CD8+ subset thereof is MHC class I restricted. In contrast, spleen cells from tumor bearing mice treated with the same amount of a fusion protein (ch225-IL2) not reacting with the tumor showed no specific lysis of tumor cells.
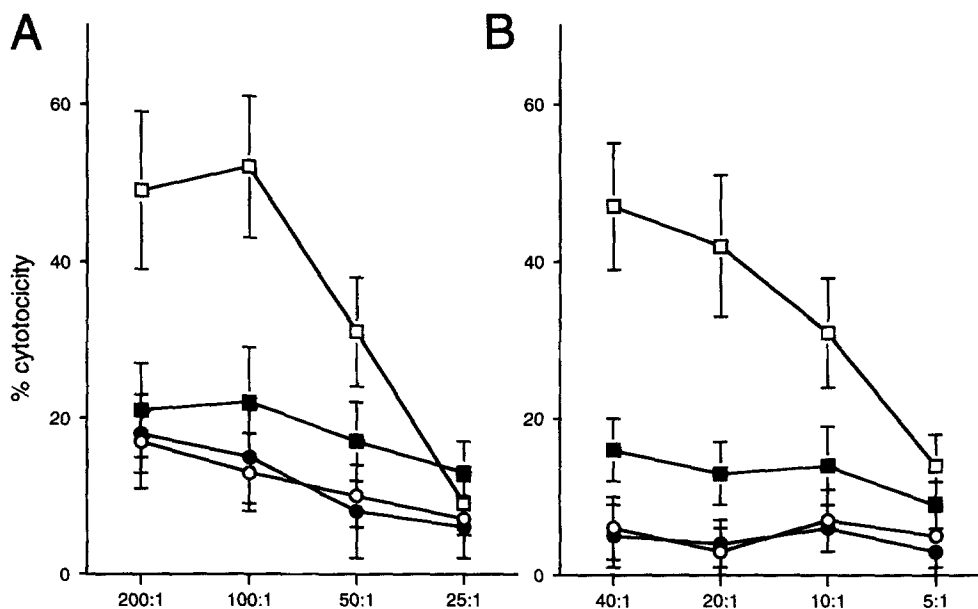

Figure 2. T cell mediated cytotoxicity against B78-D14 induced by antibody-IL2 fusion proteins. C57BL/6 mice bearing pulmonary metastases of B78-D14 melanoma cells, were treated 1 wk after tumor induction with 8 μg ch14.18-IL2 (*squares*) or nonspecific fusion protein ch225-IL2 (*circles*) for 7 d. Splenocytes (*A*) or CD8+ cells (*B*) were isolated 3 d after cessation of therapy and analyzed for their lytic activity against B78-D14 cells. Experiments were performed in the absence (*open symbols*) or presence (*closed symbols*) of an excess (50 μg/ml) of antibodies directed against H-2Kᵇ/H-2Dᵇ MHC class I antigens (clone 28-8-6, C3H IgG₂ₐ, κ). Percentage of specific lysis is plotted on the y-axis for various effector to target rations. Each value represents the mean and standard deviation for three animals.

Discussion

Antibody-cytokine fusion proteins combine the unique targeting ability of antibodies with the multifunctional activity of cytokines. In the present report we demonstrate the therapeutic effectiveness of such constructs for the treatment of established hepatic and pulmonary melanoma metastases. Several lines of evidence, i.e., in vivo depletion studies and in vitro cytotoxicity assays, indicate that this antitumor effect is largely dependent on CD8+ T cells, which kill the tumor cells in an MHC class I–restricted manner.

Since becoming available in recombinant form, IL2 has been used as an in vivo T cell growth factor in the treatment of patients with advanced renal cell carcinoma or melanoma (4). The aim of this partially successful approach is to generate or propagate tumor-reactive lymphocytes. Forni et al. demonstrated that injection of a physiological dose of IL2 directly into tumors caused suppression of their growth (9). The major advantage of an in situ application is that it avoids certain forms of toxicity associated with the systemic use of cytokines. Recently, in situ cytokine therapy has been developed further by transferring cytokine genes into tumor cells (2, 3). We reasoned that by using the targeting ability of tumor-specific monoclonal antibodies we could develop a technically more simple strategy to achieve effective concentrations of IL2 in the tumor microenvironment (5). We demonstrate here that this approach is capable to induce and maintain effective IL2 concentrations at the tumor site. Analysis of the biodistribution of ^{125}I-ch14.18-IL2 demonstrated its localization in tumor bearing livers and lungs. Therefore, it seems feasible to achieve effective local cytokine concentrations at the tumor site in a non personalized way, which makes this approach more practicable for clinical applications than the ex vivo transfer of cytokine genes. Furthermore, antibody-targeted IL2 therapy is essentially different from such gene transfer approaches with regard to the order of events; the latter aims at the induction of an antitumor host immune response by re-inoculation of IL2-producing tumor cells, whereas antibody-IL2 fusion protein therapy direct IL2 to the microenvironment of established tumors. This is particularly noteworthy, not only because of the high clinical relevance of this setting, but also because it might change immune reactions in several ways. The host immune system has most likely encountered the tumor before the immune modulation is initiated. Therefore, priming of T cells may have already occurred via antigen presenting cells, e.g., macrophages or dendritic cells; especially, since tumor cells are more sensitive to the innate cytodestructive effects of these cells immediately after inoculation than after establishment in micrometastases. Another possibility is that some tumor cells might have metastasized to the draining lymph node during this period. This would overcome the problem that naive T cells are compartmentalized in blood and lymphoid organs (10).

Taking these considerations into account, there are a number of possible mechanisms by which antibody-IL2 fusion proteins can induce eradication of disseminated tumor metastases. First, the tumor cells themselves might interact with naive T cells with IL-2 acting as the second costimulatory signal in the activation of cytotoxic T cells. A recent model proposed by J. Sprent for the activation of naive T cells provides the rationale for this mechanism (11) . According to this model, high-avidity interactions between peptide-MHC class I complexes and the T cell receptor promotes strong cross-linking of T cell receptor-CD3 complexes, which in turn leads to strong signaling; thereby stimulating the production of cytokines, such as IL2, and receptors thereof; costimulation boosts the T cell receptor mediated signal. If the intensity of signaling is below a certain threshold, e.g., when the density of peptide-MHC complexes or the level of costimulation is low, the responding T cells express IL2 receptors, but no IL2. Hence, these T cells fail to proliferate unless exposed to exogenous IL2. The second possible scheme for the establishment of T cell activation is based on tumor antigens being processed by antigen-presenting cells that transfer to the lymph node, where T cell priming then occurs. It has been shown that preactivated macrophages, dendritic cells and granulocytes express receptors for IL2 and that in vitro culture with IL2 causes functional changes in these cells (12, 13). After arriving at the tumor site these cells may be activated by the antibody-targeted IL2 to kill these tumor cells and subsequently present the tumor antigens to T cells. One further possibility is that the CD8+ T cells act as non-specific IL2-activated killer cells. However, since the applied amounts of IL2-activity are too low to induce a generalized LAK cell phenomenon (4), and application of a tumor-unspecific fusion protein failed to show any therapeutic effect or to induce B78-D14 cytolytic cells, this possibility is rather unlikely. Furthermore, in vitro cytotoxicity studies confirmed the MHC class I restriction of the antibody-IL2 fusion protein-induced immune response.

In conclusion, we have demonstrated the efficacy of antibody targeted-IL2 in the treatment of established, disseminated melanoma metastases affecting clinically relevant organs. The fusion protein-induced eradication of the tumor is critically dependent on T cells, and is likely due to either priming of naive T cells, the activation of cytotoxic effector cells, or both.

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