ORIGINAL ARTICLE

Tumor-derived interleukin-10 as a prognostic factor in stage III patients undergoing adjuvant treatment with an autologous melanoma cell vaccine

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Abstract

Objectives Interleukin-10 (IL-10) downregulates T-cell-mediated immune responses. We studied the association between IL-10 production by freshly isolated melanoma cell suspensions in vitro and overall survival in patients undergoing adjuvant treatment with a vaccine prepared from the same autologous melanoma cells modified with a hapten, dinitrophenyl (DNP).

Methods Forty-four patients with cutaneous melanoma (29 stage III and 15 stage IV) were prospectively evaluated. Tumor cells were extracted from metastatic deposits for production of DNP-modified autologous melanoma cell vaccine. Small aliquots of the melanoma cell suspensions were separated prior to vaccine processing and cultured overnight for IL-10 production. Based on a blind assessment of the distribution of IL-10 levels in the culture supernatants, a cutoff of 200 pg/ml was used to define high versus low IL-10 producers. Cox regression model was used for multivariate analysis. Overall survival was calculated using the Kaplan–Meier method, and survival curves were compared with the log-rank test.

Results Out of 44 patients, 29 were low and 15 were high IL-10 producers. The median OS was significantly worse for high compared with low IL-10 producers (10.5 months vs. 42 months; $P = 0.022$). In stage III patients, the multivariate hazard ratio for high versus low IL-10 producers was 2.92 (95% CI, 1.04–8.20; $P = 0.041$). The corresponding hazard ratio in stage IV patients was 0.92 (95% CI, 1.04–8.20; $P = 0.888$).

Conclusions High IL-10 production in the tumor microenvironment could be a determinant of clinical outcomes in stage III melanoma patients receiving autologous melanoma cell vaccine.

Disclaimer: David Berd owns stocks of AVAX Technologies.

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Introduction

The cytokines produced in the tumor microenvironment are key factors in modulating an antitumor immune response induced by active specific immunotherapies such as a cancer vaccine. We have reported that mRNA of interleukin 10 (IL-10) was present in metastatic melanoma tissues, and IL-10 was produced by melanoma cells freshly isolated from metastases [1]. Furthermore, serum IL-10

levels correlated with disease progression of patients with metastatic melanoma [1–3].

Elevated IL-10 expression has been demonstrated in various human tumors including melanoma, ovarian cancer, head and neck cancer, renal cell carcinoma, colon cancer, lymphoma and myeloma [1, 4–12]. Elevated serum IL-10 levels have been reported as a negative prognostic factor in Hodgkin and non-Hodgkin lymphomas [13, 14]. Detectable serum levels of IL-10 have also been demonstrated to be associated with poor clinical outcomes in other tumors including hepatocellular cancer, renal carcinoma, lung cancer, gastric, and colorectal carcinoma [15–18]. Preclinical data suggest that IL-10 in the tumor microenvironment plays a critical role in tumor progression and metastases [8].

In this study, we investigated the correlation between the clinical outcomes of melanoma patients treated with autologous cancer vaccine and the IL-10 levels in their tumor specimens used for cancer vaccine production. We hypothesized that the production of IL-10 by melanoma cell suspensions used for cancer vaccine would reflect the IL-10 levels in the tumor microenvironment [1, 5]. We further hypothesized that high IL-10 production in the tumor microenvironment is detrimental to the efficacy of cancer vaccine.

Materials and methods

Patients

The study population consisted of 44 consecutive melanoma patients who received an autologous DNP-modified melanoma cell vaccine between October 1995 and April 1996. After the surgical resection of their metastasis, all patients received a vaccine consisting of autologous melanoma cells modified with a hapten, dinitrophenyl (DNP). The DNP-modified melanoma cell cancer vaccine study was approved by Institutional Review Board at Thomas Jefferson University. Informed consent was obtained from all the patients participating in the study. Patients were entered onto the protocol within 30 days of surgical removal of their metastasis. The major entry criteria were as follows: sufficient amount of tumor specimens for vaccine processing (≥2.5 cm); excellent performance statues (Karnofsky score ≥70); age ≥16 years; no systemic anti-melanoma therapy within 8 weeks of study entry; and not taking corticosteroids or other immunosuppressive drugs.

Cancer vaccine preparation and treatment

The method of preparing autologous, DNP-modified vaccine and the schedule of administration of vaccine have

been previously described [19, 20]. In brief, tumor cells were extracted by enzymatic dissociation of freshly obtained metastatic melanoma with collagenase and DNAase, aliquoted, frozen in a controlled-rate freezer, and stored in liquid nitrogen in a medium containing 2.5% human albumin and 10% dimethylsulfoxide until needed. On the day of treatment, an aliquot of tumor cells was thawed. Small sample of tumor cells was separated, cultured overnight, and then assayed for the presence of IL-10 in the culture supernatants. The rest of the cells for vaccine production were washed and irradiated to 25 Gy. Then, the cells were washed again and modified with DNP. This involved a 30-min incubation of tumor cells with dinitro-fluorobenzene (DNFB), followed by washing with saline. Each vaccine fell within a specified dose range of live (trypan blue–excluding) tumor cells suspended in 0.2 ml of Hanks solution with human serum albumin. The final (haptenized) vaccine product ($2.5–7.5 \times 10^6$ cells) did not produce any IL-10. Cyclophosphamide 300 mg/m^2 was administered 3 days prior to the first vaccine injection. The vaccine product was mixed with a titrated dose of Bacille Calmette–Guérin (BCG) and injected intra-dermally on a weekly basis for 6 times. A booster vaccine was given at 6 months if patients had no sign of recurrence. After the completion of 6 weekly vaccine treatments, patients were evaluated by history and physical examination every 2 months in years 1 and 2, every 3 months in year 3, every 6 months in years 4 and 5 and yearly thereafter. All patients were followed for a minimum of 5 years or until death. Overall survival (OS) of individual patient was measured from the date of surgery to their death or last date of follow-up. Patients who were alive at the time of analysis or lost to follow-up were censored.

Delayed type hypersensitivity (DTH) response to melanoma cells

Prior to and after completion of 6 weekly vaccines, patients were tested for DTH to modified and unmodified vaccine consisting of autologous melanoma cells. Skin test materials were injected intra-dermally into the ventral forearm. After 48 h, the maximum diameter of skin induration was measured. A positive response was defined as a maximum diameter of induration ≥5 mm.

Measurement of IL-10 levels

For IL-10 production, tumor-derived cells were counted under microscope and cell numbers were adjusted to 10^6 live tumor cells/ml based on size and morphology of cells and exclusion of dead cells by trypan blue. Two hundred microliter of tumor cell suspensions (2×10^5 tumor cells) was placed in triplicate in a 96 flat-bottomed microtiter

plate in RPMI (Mediatech, Washington, DC) supplemented with 10% fecal calf serum, 4 mM L-glutamine, 10 mM HEPES, 1% non-essential amino acid, penicillin G at 100 U/ml, and streptomycin at 100 μg/ml. Tumor cells were cultured at 37°C in 5% CO_2 incubator over night (18 h), and supernatants were collected and stored at −20°C. IL-10 levels were measured by commercially available validated ELISA kit for human IL-10 (Endogen, Cambridge, MA). The sensitivity of this assay is 3 pg/ml. There is no cross-reactivity of this kit with other human cytokines. The mean of IL-10 levels in triplicate was used as IL-10 level for each specimen. Standard deviations of the test results in these triplicates were less than 10% of the means. In patients whose tumor specimens were tested for IL-10 production on multiple occasions, median IL-10 level was used for study analysis.

Statistical analysis

All analyses were performed using SAS (SAS Institute, Inc., Cary, NC) and S-Plus 8.0 (Insightful Corp., Seattle, WA) statistical software. Descriptive statistics were used to characterize the study population. The cutoff of 200 pg/ml was used to differentiate high versus low IL-10 producers based on the histogram (Fig. 1). The histogram of the IL-10 levels suggested that the distribution of IL-10 levels in these data may be viewed as a mixture of two subpopulations with different location and scale parameters, with 200 pg/ml level as an approximate boundary or joint point of the two subpopulations. The first subpopulation (66% of the data) is more localized and distributed on the interval (0–200), while the second subpopulation (34% of the data) has substantially higher spread over the interval

(200–1000). Since the sample size is relatively small, the IL-10 levels were dichotomized using 200 pg/ml as a cutoff for statistical analysis.
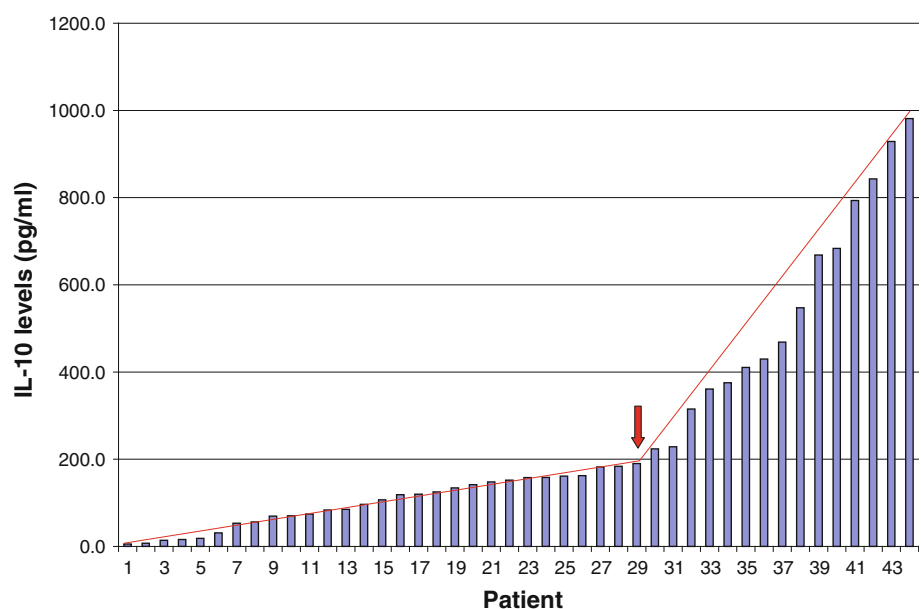
The differences between proportions of patients were tested using Fisher's exact test. Univariately, OS was evaluated using Kaplan–Meier survival curves, and differences in survival were tested using the log-rank test. Cox regression model was used to estimate multivariable-adjusted hazard ratios (HR) and 95% confidence intervals (95% CI). The final covariates used in the multivariate analysis were IL-10 levels, age, sex, stage, and the interaction term between stage and IL-10 levels. The model incorporated age (dichotomized as ≥60 years vs. <60 years) and gender as known prognostic factors. Proportional hazard assumptions were appropriate overall and for each covariate. The two-sided P-value of 0.05 was used for statistical significance.

Results

The study population consisted of 29 stage III and 15 stage IV cutaneous melanoma patients treated with an autologous DNP-modified vaccine. The 29 stage III patients had undergone complete resection of their metastases. Two out of 15 patients with stage IV melanoma had no clinical evidence of disease after the surgery.

Although single cell melanoma suspensions were made from the same melanoma metastasis, the production of IL-10 by individual cell suspensions would be variable due to minor differences in cell preservation condition and process of vaccine production. Therefore, we tested individual melanoma cell suspensions that were obtained from the same batch of tumor specimens and used for vaccine

Fig. 1 Production of IL-10 by tumor cell suspensions. The *arrow* denotes the cutoff point (IL-10 level of 200 pg/ml) to differentiate low versus high IL-10 producers

production as long as sufficient aliquots of tumor cells suspensions were available for IL-10 measurement (median of 4 tumor cell suspensions, range 1–5). The median IL-10 level of single cell suspensions from the same batch of tumor specimens was used for study analysis. Multiple (4 or 5) measurements of IL-10 production were available for 34 patients. From the linear mixed effects model fitted to repeated log-transformed IL-10 measures with the random effect of patient, it was estimated that the mean log-transformed IL-10 level was 4.83 (125 on the original scale), within-patient standard deviation of log-transformed IL-10 measures was 0.71, and between-patient standard deviation of log-transformed IL-10 measures was 1.27. Thus, the coefficient of variation associated with multiple within-patient measures is 0.147.

Levels of IL-10 produced by freshly isolated melanoma cell suspensions ranged from 5.6 to 981.3 pg/ml with the median of 154.9 pg/ml and mean of 254.2 pg/ml. As described in the statistical analysis section, an IL-10 level of 200 pg/ml was taken as a cutoff to differentiate low and high IL-10 producers. By this definition, 29 patients (66%) were low IL-10 producers (<200 pg/ml) and 15 patients (34%) were high IL-10 producers (≥200 pg/ml). The median follow-up time was 125 months (10.4 years). Baseline characteristics of the study population are summarized in Table 1. The differences in the baseline characteristics were not statistically significant in two groups. Low IL-10 producers had a higher proportion of patients with stage III disease (72% vs. 53%), although this is not statistically significant ($P = 0.315$). Additionally, the

cohort of IL-10 low producers included more patients with ages older than 60 years, compared with those with high IL-10 producers (38% vs. 20%, respectively, $P = 0.314$). The median age of patients in the low and high IL-10 producers was 55 years (range: 23–81 years) and 57 years (range: 34–75 years), respectively. Seven patients (24%) in low IL-10 producers and 2 patients (13%) in high IL-10 producers group had received prior treatment with chemotherapy, radiation, interferon, immunotherapy, or a combination thereof ($P = 0.695$). In stage III melanoma patients, there were no significant differences between the two groups with respect to the original site of tumor and nodal status.

The univariate analysis of the potential prognostic variables indicated that the stage of the disease was the major determinant of survival (Table 2). The median OS of patients with stage III and stage IV disease was 55.8 months and 12.3 months, respectively ($P < 0.001$). The DTH response to unmodified autologous melanoma cells was not a significant prognostic determinant of OS, although there is a tendency of better survival in patients who developed a positive DTH response to melanoma cells after vaccine treatments. The levels of tumor-derived IL-10 are also significant prognostic factor for survival. The median OS was significantly longer for low IL-10 producers as compared with high IL-10 producers (42 months vs. 10.5 months, $P = 0.022$). Figures 2 and 3 demonstrate the Kaplan–Meier survival curves stratified by levels of

Table 1 Baseline characteristics of the patients

	Low IL-10 producers	High IL-10 producers	P-value
Number of patients	29	15	
Median age (range), years	55 (23–81)	57 (34–75)	
Age			
≥60 years	11 (38%)	3 (20%)	0.314
<60 years	18 (62%)	12 (80%)	
Sex			
Male	17 (59%)	10 (67%)	0.748
Female	12 (41%)	5 (33%)	
Stage			
III	21 (72%)	8 (53%)	0.315
IV	8 (28%)	7 (47%)	
Prior therapy	7 (24%)	2 (13%)	0.695
DTH response			
≥5 mm	15 (52%)	11 (73%)	0.299
<5 mm	11 (38%)	3 (20%)	
Unknown	3 (10%)	1 (7%)	

DTH delayed type hypersensitivity

Table 2 Univariate analysis of effect of variables on OS

Parameter	Median OS (months)	95% CI	P
Age			
≥60 years	15.1	10.5–84.0	0.663
<60 years	19.0	9.7–56.5	
Sex			
Male	13.6	9.7–52.6	0.169
Female	20.2	12.3 to NR	
Stage			
III	55.8	13.1 to NR	<0.001
IV	12.3	7.7–17.9	
Prior therapy			
Yes	13.7	4.7–18.5	0.294
No	20.2	12.3–56.5	
DTH response			
≥5 mm	26.7	13.1–84.0	0.395
<5 mm	15.7	7.5–55.8	
IL-10 levels			
≥200 pg/ml	10.5	7.5–13.1	0.022
<200 pg/ml	42.0	13.7 to NR	

OS overall survival, DTH delayed type hypersensitivity, NR not reached
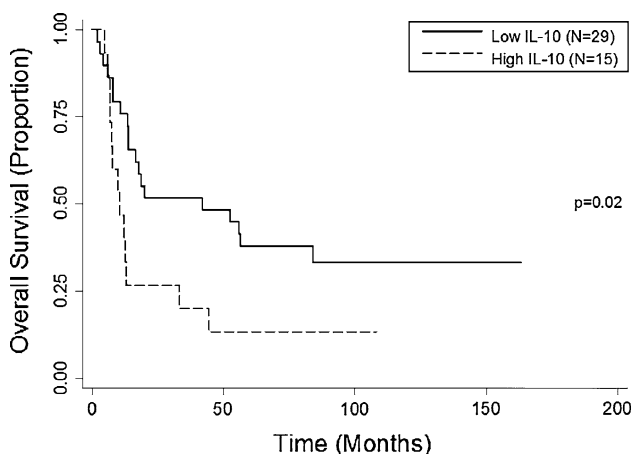
Fig. 2 Overall survival of patients treated with autologous, DNP-modified vaccine stratified by IL-10 levels
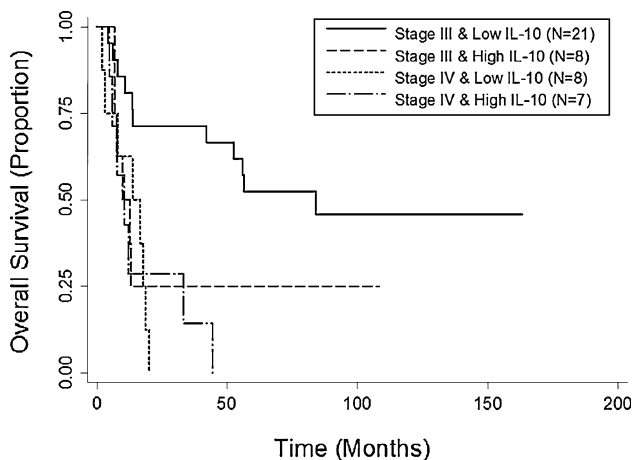

Fig. 3 Overall survival of melanoma patients stratified by stage and IL-10 levels. The *P*-values for the differences in survival between low and high IL-10 producers in stage III and stage IV patients were 0.073 and 0.624, respectively

IL-10 in tumor cell suspensions. It is of note that the impact of tumor-derived IL-10 on survival was more prominent in stage III patients, although the difference was not statistically significant (median OS for low IL-10 producers and high IL-10 producers was 84 months and 9.7 months, respectively; $P = 0.073$). For stage IV patients, there was no significant difference between low and high IL-10 producers (13.7 months vs. 10.5 months, $P = 0.624$).

To further investigate the impact of tumor-derived IL-10 on OS, a multivariate analysis was performed using Cox proportional hazard model (Table 3). The univariate analysis suggested possible association between IL-10 levels and stage. Therefore, the Cox model incorporated interaction between the stage and IL-10 level. For stage III patients, the HR for high versus low IL-10 producers was 2.92 (95% CI: 1.04–8.20, $P = 0.041$). In stage IV patients, the corresponding HR of 0.92 (95% CI: 0.28–3.00, $P = 0.888$) was not statistically significant. Furthermore, the difference in OS between stage III and IV is only seen in low IL-10 producers. In low IL-10 producers, the HR for stage IV versus stage III was 4.95 (95% CI: 1.74–14.09, $P = 0.003$). In contrast, the corresponding HR for stage IV versus III in high IL-10 producers was 1.56 (95% CI: 0.51–4.81, $P = 0.440$).

Discussion

Therapeutic vaccines for the treatment of cancer aim to induce or boost T-cell immunity against tumor-specific or tumor-associated antigens. It is the general consensus that cancer vaccine could elicit a T-cell immune response against cancer antigens that were used for vaccine treatment [21]. However, there is significant discrepancy between development of systemic immunity against targeted antigens and clinical outcome of cancer vaccine treatments. Furthermore, the presence of vaccine-elicited cytotoxic T lymphocytes in tumors expressing the targeted epitope also has been demonstrated without tumor regression [22]. It has been speculated that the discrepancy between increased numbers of tumor-specific T cells after cancer vaccine administration and the lack of clinical response might be explained by the interaction between cancer cells and the host immune system at the tumor site. Our current study indicates that tumor-derived IL-10 might be one of the factors to hamper the efficacy of cancer vaccine.

IL-10 has been proposed to modulate antitumor immune response by itself or in conjunction with other cytokines

Factor	Hazard ratio	95% CI	P
Age ≥60 years versus age <60 years	1.23	0.54–2.83	0.621
Men versus women	1.79	0.81–3.95	0.151
Stage IV versus III in high IL-10	1.56	0.51–4.81	0.440
Stage IV versus III in low IL-10	4.95	1.74–14.09	0.003
High IL-10 versus low IL-10 in stage III	2.92	1.04–8.20	0.041
High IL-10 versus low IL-10 in stage IV	0.92	0.28–3.00	0.888

Table 3 Multivariate analysis of variables

such as transforming growth factor (TGF)-beta. IL-10 is known to play a role in downregulating the expression of Th1 cytokines, thus augmenting the immunosuppressive activity of TGF-beta [23, 24]. IL-10 suppresses the T-cell immune response against melanoma cells by downregulating the expression of MHC class I, class II, and ICAM-1 [8]. IL-10 also downregulates co-stimulatory molecules such as CD 40 expression on dendritic cells in tumor microenvironment and suppresses their maturation and functions [25]. Dendritic cells derived from patients with progressive metastatic melanoma induce anergy in helper T cells [26]. Overexpression of IL-10 in IL-10 transgenic mice has been demonstrated to limit the ability of these mice to control the growth of immunogenic tumors [27]. Furthermore, IL-10 has an inhibitory effect on CTLA-4 blockade for tumor-eradicating immunity. It has been reported that anti-CTLA-4 antibody alone may not provide therapeutic benefit in patients with a tumor that produces IL-10 [28]. These experiments support the critical role of IL-10 in the tumor microenvironment in modulating antitumor immune response.

In this study, high IL-10 levels in metastatic melanoma tissues are associated with poorer survival in stage III melanoma patients treated with DNP-modified autologous melanoma cell vaccine. However, the association between IL-10 levels and survival was not seen in stage IV patients. Tumor burden is important in immunotherapy, and it is possible that vaccine itself might not have been effective in stage IV patients who had a residual clinically detectable tumor. In fact, 87% of stage IV patients had radiologically or clinically detectable disease after the surgery in this study. Since most patients died from progression of disease within 2 years, the therapeutic effect of vaccine was likely modest in stage IV patients. It is also possible that due to fewer number of stage IV patients in this study, the influence of IL-10 could not be accurately evaluated. Additionally, the multivariate analysis indicates that difference in survival between stage III and IV patients treated with DNP-modified autologous melanoma cell vaccine was only seen in low IL-10 producers. The better survival in stage III patients, compared with stage IV patients, was not seen in high IL-10 producers. This observation may suggest the detrimental effect of IL-10 in stage III patients who were treated with DNP-modified melanoma cell vaccine. Our study indicates that the tumor-derived IL-10 would affect the efficacy of autologous melanoma cell vaccine in stage III patients although appropriate control (patients not receiving vaccine) should be included in a future large-scale clinical trial to support this conclusion.

The majority of patients in this study had one large tumor (>2.5 cm) removed for the vaccine production. Furthermore, stage III patients were clinically disease free after surgical removal of lymph node metastasis; therefore, we were not able to test various tumor specimens from the same patients. It would be possible that the production of IL-10 might be different in tumors in a different location; however, we assume that the tendency of IL-10 production (high or low) would be most likely similar since they are synchronous systemic metastases from the same primary cutaneous melanoma.

The ability of IL-10 to suppress antitumor immunity is confounded by observations indicating that IL-10 may promote antitumor immunity under other conditions in animal models [26, 29, 30]. Despite conflicting data obtained from artificially induced IL-10 in mice models, our clinical observations [1] as well as number of other studies indicate that serum IL-10 levels are correlated with adverse clinical outcomes in patients with melanoma and other tumors [8, 13–18]. Our current study also supports the potential detrimental effects of tumor-derived IL-10 on autologous melanoma cell vaccine.

In summary, we have demonstrated first in human that tumor-derived IL-10 might predict the outcome of autologous cancer vaccine. Our data suggest that the effect of autologous vaccine in stage III melanoma patients may be enhanced by addition of an antagonist of IL-10. In fact, we have developed immunoadhesins for IL-10 by combining the extracellular domain of human IL-10 receptor 1 with the Fc regions of human IgG1 heavy chain [31]. The IL-10 immunoadhesins efficiently block the biologic activity of human IL-10 produced by melanoma cells in vitro. The IL-10 immunoadhesins can be administered either intratumorally or systemically to facilitate the induction of antitumor immune response after cancer vaccine. The blocking of IL-10 in tumor microenvironment itself may also be a useful therapeutic strategy in melanoma patients. However, due to the complexity of biological effects of IL-10 in the immune system, this strategy should be tested in carefully designed preclinical and clinical models.

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